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Laboratory Investigation of the Pathogenesis and Vector Ecology of Canine Infection With Ehrlichia Platys.

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LABORATORY INVESTIGATION OF THE PATHOGENESIS AND VECTOR ECOLOGY OF CANINE INFECTION WITH *EHRLICHIA PLATYS*

A Dissertation

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by
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ABSTRACT

Dogs were inoculated with Ehrlichia platys and either examined morphologically or infested with Rhipicephalus sanguineus (Acari: Ixodidae) ticks for studies of disease pathogenesis and vector transmission. Dogs inoculated with Ehrlichia platys were sequentially necropsied at 1 week intervals from 7 to 35 days post inoculation. Ehrlichia platys-infected dogs had generalized lymphadenomegaly and histopathologic evidence of lymphoid hyperplasia and proliferation of resident tissue macrophages in lymph nodes, liver, and spleen. Most dogs had mild hemorrhage or edema in multiple organs, including splenic intrafollicular hemorrhages in all dogs except one. Livers had hepatocellular vacuolation in addition to portal lymphangiectasia and Kupffer cell hyperplasia. All dogs developed thrombocytopenia and had increased numbers of megakaryocytes in bone marrow and spleen. Ultrastructurally, dogs had electron dense deposits associated with pulmonary interalveolar septal basement membranes, and intravascular coagulation and intracellular ehrlichial organisms were observed rarely. Increased activity of serum alanine aminotransferase and alkaline phosphatase occurred in dogs at 7 day post inoculation. The lesions caused by Ehrlichia platys were similar to those observed in dogs acutely infected with Ehrlichia

canis.

Laboratory maintained Rhipicephalus sanguineus nymph ticks were infested on Ehrlichia platys-infected dogs during initial parasitemia and thrombocytopenia. Molted Ehrlichia platys exposed adult ticks were studied by infesting susceptible dogs to assess vector capability, and by examination of tick midgut and salivary gland by light and transmission electron microscopy and immunocytochemistry. Ehrlichia platys organisms were not detected in exposed ticks, and ticks did not pass Ehrlichia platys infection to susceptible dogs. Rhipicephalus sanguineus may not transmit Ehrlichia platys infection.

Avidin-biotin-immunoperoxidase immunocytochemical stains were developed for detecting antigens of Ehrlichia platys and Ehrlichia risticii. Formalin fixation was shown to inactivate ehrlichial antigens in infected cells and tissues. Acetone fixation allowed detection of ehrlichial antigens with the immunocytochemical techniques in infected cell smears and in paraffin-embedded tissue sections. Ehrlichia platys antigens were not detected in tissues from infected dogs by immunocytochemical staining.

INTRODUCTION

Ehrlichia platys is an obligate intracellular rickettsia that infects canine blood platelets. Ehrlichia platys causes a recently recognized disease known as infectious canine cyclic thrombocytopenia about which little has been written. Little information is available on Ehrlichia platys lesion pathogenesis. No information is available regarding mode of infection, transmission, and organism maintenance in nature. Ehrlichia platys presented an opportunity for comparative study of platelet infectious disease. An experiment was designed to meet several objectives. To:

1) Determine whether or not Ehrlichia platys infection caused lesions in acutely infected dogs as evaluated by sequential necropsy, histopathology, and ultrastructural cytopathology.
2) Evaluate the potential for Rhipicephalus sanguineus to serve as biologic vector of Ehrlichia platys by arthropod feeding interanimal transmission, morphologic, and immunocytochemical studies using laboratory maintained ticks.
3) Apply an immunocytochemical staining technique to detect antigens of Ehrlichia platys in infected dog tissues.

CHAPTER I

Literature Review

Ehrlichia platys, (E. platys), is a member of the family Rickettsiaceae.[1] Rickettsiaceae have world-wide distribution and cause disease in animals and human beings.[2] Rickettsiaceae includes Rickettsia rickettsii (causative agent of Rocky Mountain spotted fever), R. tsutsugamushi (scrub typhus), R. prowazekii (louse-borne typhus), R. typhi (murine typhus), R. conorii (boutonneuse fever), Rochalimaea quintana (trench fever), Coxiella burnetti (Q fever), and Anaplasma marginale (bovine anaplasmosis). The tribe Erhlichieae of the family Rickettsiaceae includes E. platys (infectious canine cyclic thrombocytopenia), E. canis (tropical canine pancytopenia or canine erhlichiosis), E. equi (equine ehrlichiosis), E. sennetsu (human sennetsu rickettsiosis), E. risticii (Potomac horse fever or equine ehrlichial colitis), Neorickettsia helminthoeca (salmon poisoning), and Cowdria ruminantium (heartwater).[1,2,3] Additional members of the genus Ehrlichia include E. phagocytophila, E. bovis, and E. ovina.[1]

A general characteristic of Rickettsiaceae is that both mammals and arthropods are natural hosts.[2] the Rickettsiaceae possess common characteristics that are intermediate between bacteria and viruses. Their

resemblance to viruses is their ability to grow and replicate only within eucaryotic cells. Bacteria-like characteristics predominate and include multiplication by transverse binary fission, presence of both RNA and DNA, possession of enzymes of the Kreb's cycle, electron transport, and protein syntheses; and the inhibition of their growth by antibacterial agents.[2]

Rochalimaea quintana is the single exception to the obligate intracellular fastidiousness of Rickettiaceae, because it grows extracellularly in its invertebrate host and on artificial media.[3] Rickettsia rickettsii is capable of growth only within the nucleus and cytoplasm of host cells, such as mammalian endothelial cell,[4] or several cell types of ticks.[5] Even though these growth characteristics are different, R. rickettsii has similar nucleic acid hybridization pattens to Rochalimaea quintana.[6] Further, the sequence of nucleic acids in Rochalimaea quintana is similar to Agrobacterium tumefaciens and other purple bacteria (alpha subdivision of eubacteria), which are generally associated with plants rather than animal disease. Nucleic acid sequencing suggests a lineage may exist between plant associated purple bacteria and rickettsiae. Evolutionarily then, the Rickettsiaceae may have roots as plant pathogens, later becoming associated with animals through an arthropod-vector bridge.[6]

Members of the genus Ehrlichia are small (0.4-4.0 um diameter), pleomorphic, gram-negative, coccoid to ellipsoid organisms. The ehrlichiae which are pathogenic for mammals occur within cytoplasmic vacuoles either singly or as multiple inclusions forming a cluster (morula).[1,7] Morphologic studies indicate that Ehrlichia spp. occur within a phagosome, believed to be derived from host cell plasma membrane, and reproduce there by binary fission.[6,7,8] Ehrlichia spp. differ in their target cells and hosts. E. platys infects platelets. E. platys differs from other ehrlichiae infective for dogs since E. canis infects monocytes and lymphocytes, and E. equi infects canine and equine granulocytic leukocytes.[7] The mammalian host range of the tribe Ehrlichieae includes most domestic animals (cats and pigs may be exceptions),[1,9] certain wild canidae such as wolves, coyotes, or foxes;[10] and human beings, in the case of E. sennetsu and possibly E. canis.[11] Some of the Ehrlichieae have been shown to be adapted to biologic transmission in an arthropod, similar to other Rickettsiaceae.[1,9]

Ehrlichia platys was described by Harvey and others[7] in 1978, in a syndrome referred to as infectious canine cyclic thrombocytopenia (ICT).[12] The organism was observed within 17% of platelets during routine examination of a peripheral blood smear from a 1 year-old

female Keeshond dog. The organism appeared as single or multiple basophilic inclusions in platelets. The dog was thrombocytopenic (33.0 X 10^3 platelets/ul) and mildly anemic (hematocrit 32%), while other hematologic parameters were considered within reference ranges.[7]

The ultrastructural morphology of E. platys within the vertebrate host is similar to other rickettsiae such as E. canis,[7,13] Anaplasma marginale (A. marginale),[14] and Cowdria ruminatium.[15] Each individual inclusion, also referred to as a subunit, is surrounded by a double membrane, the outer of which is often ruffled or finely undulated. These double membranes surround a mixture of granular and fibrillar material comprising the internal portion of the subunit that has an affinity for uranyl acetate stain. Such staining suggests that these components are RNA and DNA nucleoprotein.[14] Furrow-shaped indentations in some "bean"-shaped organisms are interpreted as evidence of reproduction by binary fission. Individual subunits or clusters (morula) of up to eight subunits are surrounded by a single membrane that is usually in close apposition to subunit double membranes. These represent a microorganism with diameter ranging from 0.4 to 1.2 um. Up to three morulae occur in a single infected platelet.[7]

Ehrlichia platys microorganisms have been observed only in circulating platelets, and not in marrow

megakaryocytes, marrow and extramedullary hematopoietic cells, or in circulating leukocytes or erythrocytes.[7] The method by which E. platys gains entry into platelets has not been determined. Greene and Harvey[16] show evidence of an E. platys subunit in close extracellular association with an invaginated area in a platelet plasma membrane in an electron micrograph credited to the late C. F. Simpson.[7] Greene and Harvey[16] suggest that the single outer membrane defining a microorganism may be derived from platelet plasmalemma and entry may be by endocytosis similar to the method described for A. marginale.[14]

Ehrlichia platys was classified with the Ehrlichia spp. primarily based upon its morphologic similarity to E. canis.[1] Like E. canis and the intraerythrocytic rickettsial bovine parasite Anaplasma marginale, E. platys has similar sized subunits, and similar membrane structure surrounding prokaryotic nucleocytoplasm containing DNA and RNA.[1,7,12,14] Reproduction appeared to be similar to other Rickettsiaceae.

Attempts to culture E. platys in vitro have thus far been unsuccessful.[16] Attempts to infect a cat[16] and a rabbit (Simpson RM, Gaunt SD. unpublished data, 1986) were also unsuccessful.

An indirect immunofluorescent antibody (IFA) test was developed for detection of serum antibodies to E.

platys.[12] Serologic evidence of E. platys infection through detection of anti-E. platys antibodies by IFA is presently the most practical and reliable method of diagnosis. However, a positive serum antibody titer to E. platys does not necessarily indicate current infection. Demonstration of E. platys inclusions in platelets is a more definitive diagnosis of active infection, but is difficult during episodes of thrombocytopenia when few if any organisms are present in platelets. Subsequent episodes of cyclic thrombocytopenia are preceeded by even lower concentrations of infected platelets, so chances of detecting infected platelets becomes less as the duration of infection becomes greater.[12,16] The IFA assay is specific for E. platys and does not cross-react to serum antibodies from dogs with E. canis infection or dogs with immune-mediated thrombocytopenia.[12] E. platys positive sera have been documented from dogs residing in Florida, Pennsylvania, Texas, Louisiana, Illinois, California, Arkansas, Mississippi, Idaho, North Carolina,[12] and Georgia (Lafferty RR, personal communication, 1987). Five out of 100 random source healthy dogs tested had positive anti-E. platys serum antibody.[12] Nine of 26 thrombocytopenic dogs (35%) were seropositive for E. platys and 7 of these nine dogs were also seropositive for E. canis. These serologic surveys indicate that combined infection by E. platys and E. canis may be as common as E.

canis infection alone.[12] Thus E. platys apparently has a wide geographic range in the United States, and its range may be similar to the world-wide distribution of E. canis in temperate and tropical regions.

Overt clinical signs in dogs infected by E. platys appear to be uncommon and thus far those observed are of a mild degree. The infected Keeshond dog reported from Florida[7] lacked clinical signs of illness and was presented as the result of the recent death attributed to babesiosis of one of her puppies. Dogs experimentally infected with E. platys by inoculation of blood from the bitch were only mildly febrile (39.4°C-40.2°C) and only 1 of 4 dogs had any sign of bleeding (hematochezia) during a thrombocytopenic episode. In addition, another dog in that study had a prolonged bleeding time (>30 minutes) when platelet numbers were low (2.0 X 10^3/ul).

Intrathrombocytic subunits morphologically identical to E. platys were observed in a thrombocytopenic 9-month-old Chow Chow dog presented to the Louisiana State University Veterinary Medical Teaching Hospital with uveitis.[17] The dog had a high (1:100) antibody titer to E. platys but no titer to E. canis by indirect immunofluorescent antibody tests. The presence of E. platys was further confirmed by intravenous inoculation of a splenectomized dog with heparinized blood from the Chow.

The blood had been stored frozen for 1 week at -80° C. The splenectomized dog developed hematologic changes typical of E. platys infection, but showed no clinical signs nor acquired any ocular lesions. The Chow Chow dog responded favorably to topical opthalmic antibiotic, corticosteroid, and atropine therapy as well as systemic treatment with tetracycline. Antimicrobial chemotherapy with tetracycline drugs is generally effective treatment for both E. platys and E. canis.[17] A dose of 22 mg/Kg t.i.d. has been recommended,[17] similar to dosage for acute E. canis infection in dogs.[16] One other dog from Georgia suffering from uveitis had serologic evidence of exposure to E. platys (and concurrently tested negative for E. canis by IFA) (Lafferty RR, personal communication, 1987). In addition to isolates from Florida and Louisiana, E. platys has been isolated and experimentally inoculated into dogs in Oklahoma (Ewing SA, personal communication, 1987).

Only the one or possibly two cases of uveitis associated with E. platys infection have been recorded,[17] (Lafferty RR, personal communication, 1987) leaving the impression that E. platys infection is often clinically inapparent.[16] The same can be true of E. canis, however clinical signs and even fatality are often ascribed to E. canis.[16,18] E. platys infected dogs do not have clinical bleeding tendencies, while hemorrhagic diatheses in E.

canis infected dogs can be severe and prolonged. Clinical signs more commonly associated with E. canis infection include lethargy, depression, anorexia, fever, weight loss, dyspnea, oculonasal discharge, and lymphadenopathy.[18,19] Interstitial pneumonia has been documented radiographically in E. canis infection.[16]

One milliliter aliquots of blood from the Keeshond dog from Florida,[7] were inoculated into experimental dogs intravenously. Seven to twelve days following inoculation, circulating platelets contained inclusions or morulae of E. platys visible in Wright's stained blood smears. The appearance of E. platys inclusions within platelets in Wright's stained blood smears was referred to as "parasitemia".[7] Parasitemia reached a maximum of 31-63% three to four days after inclusions initially appeared within platelets. Platelet numbers declined rapidly and reached a nadir 8-15 days post inoculation with measurable platelet concentrations in the range of 2.0-15.0 X 10^3/ul. Platelet concentrations remained low for 3 to 4 days and organisms were not observed in blood smears during this period. Platelet concentrations then returned to pre-infection levels over about 3 days. Parasitemia reappeared, followed by subsebquent thrombocytopenia. Cycles of thrombocytopenia were episodic occurring an average of every 10.5 days (range 7-14 days). Maximum

percentages of parasitized platelets occurred during initial episodes. Subsequent cycles of thrombocytopenia were preceeded by fewer parasitized platelets, with some infection rates as low as 1%.[7] The cyclic relationship of parasitemia and thrombocytopenia in E. platys infections appears to diminish with time with spontaneous resolution after several weeks or months in many cases.[7,16]

Platelet function was examined in E. platys infected dogs by studies of in vitro canine platelet aggregation stimulated by collagen and adenosine diphosphate (ADP). Platelet response to stimulation by collagen or ADP studied up to 28 days post inoculation (DPI) in dogs acutely infected with E. playts was not statistically different from uninfected dogs.[20] The pathogenesis of thrombocytopenia in dogs caused by Ehrlichia spp. is unknown. In acute E. canis infection decreased platelet survival,[21] increased cell mediated cytotoxicity,[22,23] complement activation,[22,23] and splenic platelet sequestration[24,25] may all contribute to thrombocytopenia.[16,18] The mechanism of thrombocytopenia in acute E. platys infection has been hypothesized to involve direct platelet damage.[16] There is some circumstantial evidence to suggest a direct erhlichiacidal cytolytic effect in E. canis infected monocytes.[26] Another study on E. canis failed to document this cytolysis.[8] Direct platelet damage by E. platys infection

as a sole mechanism of thrombocytopenia is unlikely, because during subsequent episodes of thrombocytopenia the numbers of measurable parasitized platelets is smaller than the loss of circulating platelets.[16]

Ehrlichiae induced thrombocytopenia may result from platelet sequestration or consumption and the cause may be multifactorial. Sequestration of infected monocytes has been reported in E. canis infected dogs.[27] Other theories of platelet consumption include antibody-mediated platelet removal;[22] complement-mediated removal,[22,28] as in E. canis infection;[22] or platelet activation secondary to endothelial damage as occurs in Rickettsia rickettsii infection.[29] Ehrlichiosis may sensitize macrophages to platelets resulting in immune-mediated phagocytosis. Alternately, changes in platelet antigens may result in immune-mediated removal or destruction.[30]

Other hematologic changes that occur in dogs acutely infected with E. platys include a normocytic, normochromic anemia that develops concurrently with the initial parasitemia.[31] This pattern most closely resembles an anemia of acute inflammation associated with decreased erythrocyte survival and disordered iron metabolism.[32,33] Decreases in serum iron and total iron binding capacity occur in E. platys infected dogs similar to decreases which occur in anemias of inflammation. Recovery from

anemia in E. platys-infected dogs occurs in about 4 weeks post inoculation.

Ehrlichia platys infected dogs also become mildly leukopenic at approximately the same interval as the anemia and parasitemia.[7,31] Decreased numbers of neutrophils, lymphocytes, and eosinophils were observed. Ratios of myeloid to erythroid series in bone marrow were increased in marrow aspirates from 7 to 14 DPI but slightly decreased 21 through 35 DPI in E. platys infection.[31] The mechanism of leukopenia is unknown, but may result from increased demand and utilization, sequestration due to increased margination, or ineffective myelopoiesis.

Changes in serum albumin, calcium, and serum protein electrophoretic patterns occur during acute E. platys infection.[31] In dogs monitored up to 35 DPI, albumin was decreased from 7 to 28 DPI, while alpha and gamma globulins became elevated. $Beta_1$ globulin fraction was mildy reduced 2 weeks PI. Total serum protein and $alpha_2$ and $beta_2$ globulins remained unchanged. Changes in these or other plasma proteins may reflect reactant changes occurring during the acute phase of inflammation.[34] Decreases in calcium that occurred in acute E. platys infection paralleled the decreased albumin. Since total measured calcium is partially bound to albumin, the decrease in calcium is probably the result of concurrent

hypoalbuminemia.[35]

Acute infection of dogs by E. platys or E. canis results in anemia, leukopenia, and thrombocytopenia. Compared to E. platys infection, the duration of thrombocytopenia in E. canis infection is variable and is not cyclic in nature. Changes in serum globulins and albumin during acute inflammation are common to infection by either Ehrlichia species.[18,31]

Few pathologic lesions have been described in E. platys infection. Uveitis was described in a naturally infected Chow Chow dog.[17] Interstitial pneumonia was the only lesion reported previously in experimental studies of E. platys infection. Interstitial thickening of interalveolar septa occurred in 2 acutely infected dogs at 2 weeks following experimental infection.[36] Interstitial pneumonia in dogs infected with E. platys for 60 days was characterized by fibrosis of interalveolar septa.[7] Several lesions occur in dogs infected with E. canis.[25,27,37] Gross lesions are limited to enlargement of lymph nodes and spleen, and reddened bone marrow. Histopathologically, generalized lymphoid hyperplasia occurs in lymph nodes and splenic follicles. Splenic follicles often contain foci of intrafollicular hemorrhage. Hyperplastic reticuloendothelial cells aggregate as multifocal nodules in liver sinusoids, that

occasionally displace small groups of hepatocytes. Hematopoietic tissue may undergo hyperplasia.[25] Interstitial pneumonia is commonly reported at necropsy.[25] Perivascular cuffs of lymphocytes and plasma cells occur surrounding renal corpusles and cortical veins, in retina, and cerebral meninges.[25,37]

The method of natural infection, transmission, and maintenance of E. platys is unknown. Many members of Rickettsiaceae utilize a variety of arthropods including lice, mites, fleas, and ticks as biologic vectors and biologic resevoir hosts. Some rickettsiae have more than one demonstrated arthropod vector.[3,38] Among arthropods as vectors of infectious disease and toxic agents, ixodid (hard) ticks are some of the most important species world-wide and their parasitic attachments have not gone unnoticed. Homer referred to the presence of ticks on Ulysses' dog. In even earlier references, Egyptian scrolls dated 1500 B.C. refer to "tick fever". The parasitic nature of ticks was recognized and recorded by Cato, Aristotle, and Pliny. Tick transmission of pathogens was first discovered by Smith and Kilbourne in 1893. They experimentally transmitted Babesia bigemina (Texas fever) with Boophilus sp. ticks.[39] Subsequent investigation led to discoveries that ticks transmit viruses, spirochetes, bacteria, and protozoa in addition to rickettsiae.[40]

Currently, three ehrlichiae have documented tick vectors. The vector for E. phagocytophila in Europe is Ixodes ricinus.[1] Ehrlichia bovis is transmitted by Hyalomma sp. ticks,[38] and the brown dog tick, Rhipicephalus sanguineus, transmits E. canis.[1] Several studies have documented transtadial transmission of E. canis, but evidence for transovarial transmission is not definitive.[9,41,42] E. canis has been experimentally transmitted by more than three strains of Rhipicephalus sanguineus (R. sanguineus) by allowing laboratory-raised ticks to feed on acutely infected dogs.[9,41] The disease was transmitted to normal beagle dogs by R. sanguineus nymphs fed as larvae, by adult ticks fed as nymphs, or by adults fed as both larvae and nymphs. The F_1 progeny from ticks transmitting E. canis as adults did not infect other normal dogs as either larvae, nymphs, or adults.[9]

The temperate and tropical planetary distribution of E. canis is believed limited only by the geographic range of R. sanguineus.[16] While R. sanguineus is the only currently known vector of E. canis, other undetermined arthropods may be capable of transmitting or may adapt to carry ehrlichiae[43]

Ticks are responsible for transmission of most varieties of the arthropod-borne diseases of domestic animals. Ticks rank second to mosquitoes as vectors of

human disease,[40] and new tick-borne diseases have recently been discovered.[11,44,45,46] Ticks are especially well suited for maintaining disease agents in nature due to the tick's hematophagus parasitic habits; the ability to survive as adults up to 3 years in an unfed, free-living state; and to lay large numbers of eggs, that in some cases can convey infectious organisms to progeny.[40,47] When ticks feed on a suitably infected host, midgut epithelial cells can become infected by organisms present in the blood meal being digested.[5] The infecting organism must remain viable in the arthropod host cell in order to be transmitted during a subsequent blood meal that may not occur for considerable time. The potential pathogen then has a complex system for survival that must be synchronized with the life cycle of its arthropod host, and little is known about these events.

A coordinated developmental cycle between the protozoans Babesia sp. and Theileria sp. and their tick hosts has been described. These organisms initially develop in gut epithelium, replicate, and subsequently penetrate salivary gland to be transmitted via the saliva when feeding recurs following a molt by the tick.[48] Other pathogens such as the Lyme disease spirochete, Borrelia burgdorferi, have recently been shown to have similar invertebrate developmental cycles.[49] The dissemination of rickettsiae, that infect acarines, to hemocoele, salivary

gland and ovary has been investigated utilizing animal inoculation,[48,53,58,61,63] animal transmission, [41,48,50-53,56,61,63] light and transmission electron microscopy,[41,48,54-63] and immunofluorescent microscopy [48,53,62] of tick tissues.

The rickettsial agent A. marginale has been observed in midgut epithelium of adult ticks fed as nymphs on infected cattle from groups of ticks that also proved to be infective by animal transmission studies.[60,62,64] The development of A. marginale in Dermacentor andersoni (D. andersoni) ticks involves several stages.[48] Midgut epithelial cells were infected in nymph ticks feeding as little as 24 hours on an infected calf.[56] A. marginale exhibited three morphologically distinct forms as observed by transmission electron microscopy in colonized nymph midguts.[65] The appearance of colonies in midgut, however, did not always correlate with infectivity of tick tissue homogenates.[48] Twenty days post-repletion tick homogenates did convey anaplasmosis to cattle, while colonies appeared in tick midgut as early as 5 days post repletion.[65] Up to 5 different types of Anaplasma rickettsial colonies occur in midgut epithelium in molted adults exposed as nymphs.[55]

Rickettsial colony development in ticks and transmission of anaplasmosis is synchronized with D.

andersoni's feeding process.[48] Rickettsial reactivation which is presumed necessary for virulence and subsequent disease transmission, is thought to occur naturally during the tick's blood meal feeding process.[41,48,52,66] Reactivation of rickettsial virulence has been simulated experimentally by incubating unfed adult ticks infected with pathogenic rickettsiae for 2.5 days at 37°C. Anaplasmosis incubation periods are shorter in cattle inoculated with homogenates of incubated unfed adult ticks than in cattle inoculated with nonincubated unfed ticks.[56] When partial feeding of infected ticks was used to stimulate rickettsial reactivation, adult ticks were required to feed for a least 6 days (total feeding time averaged 9 days) for transmission of anaplasmosis to occur. Midguts from these adult ticks fed on cattle contain few A. marginale organisms during days 7 to 9 of feeding. This suggests transmission by simple regurgitation of gut contents would be unlikely and that rickettsial reactivation occurs only at particular phases of blood engorgement.[67] Organisms have been observed in the salivary glands of feeding adult ticks during this same 7 to 9 day time period.[48] Injection of microorganisms is thought to occur with the salivary fluids during tick feeding.[41,49,59] Rickettsial infection of tick ovary, antecedant to transovarial transmission, is seen as an evolutionary adaptation providing a resevoir

for the rickettsial organism in the invertebrate host.[5,38]

Different isolates of E. canis have been transmitted both by feeding R. sanguineus ticks on naive susceptible dogs or by intravenous inoculation of such dogs with "ground-up" infected tick tissues (tick homogenates).[41,42] Transmission to noninfected dogs was successfully accomplished with as few as 24 feeding adult ticks or by inoculation of homogenates from 6 ticks at up to 155 days following repletion with a blood meal as nymphs.[42] The period from initiation of infected tick feeding or inoculation of tick tissues until appearance of clinical signs ranged from 8 to 16 days. Partial tick engorgment appeared to be desirable if not necessary for transmission of E. canis, especially when the tick tissue homogenates were used.[41] The lack of disease transmission by unfed nymphs exposed to E. canis-infected dogs as larvae was noted as a divergence from a previous study.[9]

Ehrlichia canis subunits were documented in both male and female tick midgut and salivary gland by immunofluorescent and transmission electron microscopy. In the tick, the organism morphology was similar to that of the vertebrate intraleukocytic form. Transovarial transmission did not occur, and only symbiotic rickettsiae identified as Wolbachia spp. were observed in the ovary.[41]

Rhipicephalus sanguineus transmits pathogens other

than E. canis. R. sanguineus is the most widely occurring of the ixodid ticks and can serve as a vector for a variety of infectious organisms of various taxa as well as the toxic principle of tick paralysis.[38,68] R. sanguineus may not be the usual or most significant vector of a given pathogen in each instance, as some microorganisms have more than one arthropod vector. Other Rickettsiaceae transmitted by R. sanguineus include: R. rickettsii,[16,38] Rickettsia conorii (boutonneuse fever),[38,69] a rickettsial agent of tick borne fever of cattle and goats,[68] Anaplasma marginale,[68] Coxiella burnetti thought to be transmitted in the feces of R. sanguineus,[41] and Haemobartonella canis.[70] Rhipicephalus sanguineus transmits the protozoa Babesia canis, B. gibsoni, B. caballi, B. equli,[68] and possibly Hepatozoan canis.[71] The spring-summer Russian viral encephalitis,[68] and possibly African Horse Sickness (an orbivirus),[72] are viral diseaes transmitted by R. sanguineus. Fransicella tularensis, a bacterium, may also be transmitted by R. sanguineus.[73]

The E. platys infected Keeshond bitch studied by Harvey lived in a kennel that harbored a heavy population of ticks.[7] Since serologic surveys have revealed a large number dogs that had serum antibodies to both E. platys and E. canis, coincident infection by a single common vector has been hypothesized.[12,16] Dogs with concurrent

infection by E. canis and Babesia canis have been reported, supporting the likelihood of simultaneous transmission of two organisms by a single vector.[16,27]

CHAPTER II

Lesions of Acute *Ehrlichia platys* Infection in Dogs[74]

ABSTRACT

Ehrlichia platys (*E. platys*) is a recently described rickettsial agent that infects canine blood platelets. Lesion development, not previously examined in acutely infected dogs, was studied in five purebred Beagle and five mixed-bred dogs inoculated with a Louisiana isolate of *E. platys*. Two dogs each were necropsied at each 7 day interval up to 35 days post intravenous inoculation. Tissues were collected and either formalin-fixed or frozen at -70°C. Tissue sections were examined by light, transmission electron, or immunofluorescent microscopy. Serum alanine aminotransferase and alkaline phosphatase activities were increased at day 7 post inoculation. Thrombocytopenia and *E. platys* parasitemia occurred at 10-14 day intervals in all infected dogs. All dogs subsequently developed increased serum antibody titers to *E. platys* but not to *E. canis*. Lymph nodes exhibited generalized enlargement, and microscopic lesions included paracortical lymphoid hyperplasia, sinus histiocytosis, and medullary plasmacytosis. Spleens contained marked intrafollicular areas of crescent-shaped hemorrhage and widespread nodular hyperplasia of the periarteriolar macrophage sheaths. Bone marrow and splenic red pulp

contained increased numbers of erythroid precursors and megakaryocytes. Livers contained small multifocal hyperplastic nodules of Kupffer cells, and many liver sections had hepatic edema and hepatocellular vacuolation. No lesions were observed in lungs fixed by pulmonary airway perfusion of fixative. Electron microscopy revealed multifocal thickening of interalveolar septal basement membrane in lungs and rare intravascular platelet aggregates or fibrin thrombi in lung and skin. E. platys antigen was detected by immunofluorescence in mononuclear cells in bone marrow, spleen, lymph node, and liver in frozen sections from the dogs infected for 2 weeks. The lesions caused by E. platys were similar to those observed in dogs acutely infected with Ehrlichia canis.
[Portions presented for and abstracted in Proceedings 37th Annual Meeting of the American College of Veterinary Pathologists. Infectious Diseases Specialty Group. December 5, 1986, New Orleans, LA. p. 185].

INTRODUCTION

Previous reports of morphologic lesions associated with infection by Ehrlichia platys (E. platys), the causative agent of infectious canine cyclic thrombocytopenia (ICT), conflict, involved few animals, and had other primary objectives[7,36] Experimental inoculation of dogs with E. platys results in infection of blood platelets by E. platys, with the organism visible as

inclusions or morulae (parasitemia), 7-12 days post inoculation. Parasitemia is followed by cyclic episodes of thrombocytopenia that recur at 10-14 day intervals.[7,12,17] The mechanism of thrombocytopenia in ehrlichial infection is unknown. Studies on thrombocytopenia caused by acute E. canis infection have led to several hypotheses including decreased platelet survival,[21] humoral immunity and complement activation,[16,22,23] and platelet trapping by the spleen or other resident tissue macrophages.[24,25] Evidence suggests that E. canis infects endothelium,[25,37,75] which could activate coagulation resulting in platelet consumption, similar to Rickettsia rickettsii infection.[29,76]

Normal platelet and endothelium interaction could also be altered by E. platys infection. In this study, tissues from dogs acutely infected with E. platys were sequentially examined microscopically to study the pathogenesis and to determine if lesions could be correlated with thrombocytopenia. In addition, the status of thrombopoiesis and evidence of platelet destruction were sought to elucidate the pathogenesis of thrombocytopenia.

MATERIALS AND METHODS

Ten mature healthy laboratory conditioned and

vaccinated male dogs (5 purebred Beagles, 5 mixed bred dogs) that lacked serum antibodies to E. platys or E. canis as measured by an indirect immunofluorescent antibody test (IFA) were used in this study. The dogs were inoculated intravenously with 1 ml aliquots of frozen canine platelet-rich plasma (PRP) containing 300,000 platelets/ul. Five percent of platelets contained inclusions or morulae of E. platys. Parasitemia is defined as the presence of E. platys microorganisms as inclusions (= subunits) or morulae in circulating platelets detected in Wright's stained blood smears.[7] IFA was performed using methods described in Appendix A. One mixed-bred dog inoculated with 1 ml saline served as an uninfected control. All dogs were determined to be heartworm free using Knott's test. The dogs were housed in the Louisiana State University vivarium administered according to guidelines of American Association for Laboratory Animal Care.

Serum was collected for anti-E. platys antibody determination and for enzyme analysis on 3, 7, 10, 14, 17, 21, 24, 28, 31, and 35 DPI and stored at -70^{o}C until tested. Biochemical analysis for alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activity was conducted on the 0, 3, 7, 14, 21, 28, and 35 DPI samples using an automatic centrifugal analyzer (Encore, Baker Instruments Corp.,

Allentown, PA) and commercial reagents (Baker Instruments Corp., Allentown, PA). Serum samples for antibody testing were diluted 2-fold with phosphate buffered 0.9% saline (pH 7.2) starting from 1:20 and titered to endpoint by IFA assay.[12] Whole blood was collected in sodium ethylenediaminetetraacetate anticoagulant for blood platelet quantitation and for Wright's stained blood smears. Platelet concentration was determined by diluting blood 1:1000 in ammonium oxalate solution (Unopette test 5855, Becton Dickinson, Rutherford, NJ), and multiplying by 1000 the number of platelets counted manually in the center grid of a hemacytometer chamber with the aide of light microscope (Laborolux 12, Ernst Leitz, New York, NY).

One infected beagle and one infected mixed-bred dog were euthanatized by intravenous administration of a euthanasia solution (T-61, American Hoechst Corp., Sommerville, NJ) on each of 7, 14, 21, 28, and 35 days post inoculation (DPI). The control dog was euthanatized at 35 DPI. Cadavers were examined grossly and samples of the following tissues were collected from each: eyes, brain, parotid salivary gland, thyroid gland, parathyroid glands, multiple lymph nodes (including visceral and peripheral), heart, lung, bone marrow, liver, pancreas, spleen, adrenal glands, kidneys, urinary bladder, prostate gland, scrotal skin, testes, esophagus, stomach, small and

large intestines, and skeletal muscle. Spleens were fixed by perfusing a splenic vein with 10% phosphate buffered (pH 7.2) formalin solution, followed by immersion in 10% phosphate buffered (pH 7.2) formalin solution for at least 24 hours. Additional splenic samples were fixed by simple immersion in formalin. The lungs were fixed by perfusing with a 4% phosphate buffered formalin and 1% glutaraldehyde solution (4F1G) delivered by intratracheal cannula under 20 cm high column of fixative pressure. Perfused, expanded lungs were stored at 4°C for 48 hours prior to sectioning. Skin was immersion fixed in 4F1G. Eyes were fixed by immersing intact closely trimmed globes in Zenker's fixative for 5 hours followed by overnight washing in running tap water. Fixed and washed globes were stored in dilute phosphate buffered formalin (2-5%) until processed further. All other tissue samples were immersion fixed in 10% phosphate buffered (pH 7.2) formalin for a minimum of 24 hours at room temperature (22°C). All tissues were processed routinely for histopathology using ethanol dehydration and paraffin embedding. Sections from each tissue were cut at approximately 5 um thickness and stained with hematoxylin and eosin (HE). Selected tissue sections were stained with periodic acid-Schiff stain (PAS) with and without diastase digestion, Pearl's iron, Masson's trichrome, hematoxylin basic fuschin periodic acid (HBFP), Hall's

bilirubin, modified Steiner silver, and Machiavello histochemical stains. Marrow megakaryocyte numbers were estimated by comparing mean numbers of nuclei per 5 fields at 200X.

Samples of spleen, lung and skin from one dog from each weekly examination period were sectioned (1 mm^3) and post-fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), followed by 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for transmission electron microscopy (TEM). Fixed TEM specimens were washed, dehydrated in a graded ethanol series, infiltrated and embedded in a mixture of epon and araldite epoxy resins (Electron Microscopy Sciences, Ft. Washington, PA). Semi-thin (approximately 1 um) sections were examined using light microscopy and areas for subsequent thin (60-90 nm) sections were identified. Thin sections were cut on an ultramicrotome (Sorvall MT 500, Dupont) picked up on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined using a Zeiss EM 10 electron microscope.

Separate samples of spleen, lymph node, bone marrow, liver, and kidney from each dog were frozen at -70^{o}C. Frozen sections of tissues were incubated with canine E. platys immune serum diluted 1:40 in phosphate buffered 0.9% saline (pH 7.2). Slides were washed and incubated for 30 minutes at 37^{o}C with sheep anti-dog fluorescein

isothiocyanate conjugated immunoglobulin G (Miles Scientific, Naperville, IL). Sections were examined using an epifluorescence microscope (Ernst Leitz, Wetzlar GMBH 514662).

Significant differences in platelet concentration, ALT, AST, ALP, and megakaryocyte number were determined by comparing preinfection with post-infection values with one-way analysis of variance and Duncan's multiple range test.

RESULTS

All experimentally inoculated dogs developed *E. platys* infection. Parasitemia occurred in all dogs surviving longer that 9 DPI. Mean parasitemia (5%) was maximal on 14 DPI, and both parasitemia and thrombocytopenia recurred at 10-14 day intervals. Significant decreases in mean numbers of platelets occurred at 7 DPI and reached a nadir on 17 DPI (Table 1). Mean platelet concentration increased on 24 DPI, and then decreased again at 28 DPI. Inclusions or morulae of *E. platys* were observed in platelets from 10 through 21 DPI, and again from 24 through 28 DPI (Table 1). All dogs assayed after 17 DPI had anti-*E. platys* serum antibodies by IFA, while two dogs developed titers as early as 7 DPI. Dogs had antibody titers to *E. platys* for the duration of the study. Maximum serum titers to *E. platys* ranged from 1:80 to 1:5120. Serum samples taken from all dogs

immediately prior to necropsy lacked antibodies to E. canis. The control dog remained uninfected by E. platys and serum collected prior to euthanasia at 35 DPI was negative for anti-E. platys and E. canis antibodies.

When compared to preinoculation values (day 0), the mean serum activities of ALT (preinoculation mean 28 IU/L) and ALP (34 IU/L) in the 7 DPI samples, were significantly ($P<0.05$) increased to 43 IU/L and 75 IU/L respectively. Enzyme activity in samples collected on other days was not statistically significant from preinoculation levels. Serum AST activity was not changed significantly from preinoculation values in any dogs.

At necropsy, there was generalized lymphadenomegaly in all infected dogs. Livers in the dogs examined at 7 and 14 DPI were slightly pale, mottled and swollen. Gross evidence of bleeding was not observed in any of the dogs.

Light microscopic examination of paraffin-embedded HE stained sections of lymphoid organs revealed reactive hyperplasia. Lymph nodes had hyperplastic lymphoid follicles in the cortex, with occasional formation of secondary follicles with germinal centers as early as 7 DPI. A mild increase in sinus macrophages, which occasionally exhibited erythrophagocytosis, was present in some lymph nodes. A few plasma cells were present in medullary cords. By 14 DPI, lymph nodes had marked follicular hyperplasia (Fig 1), and erythrophagocytosis by

sinus macrophages was more prominent (Fig. 2). The Beagle examined at 14 DPI had areas of hemorrhage in lymph node cortical follicles and medullary cords. Hemosiderin-laden macrophages were also present in cortex and medulla of 14 DPI lymph nodes. Plasma cells were slightly more numerous in medullas of 14 DPI dogs than in 7 DPI dogs. Hyperplastic lymphoid follicles were less striking in dogs examined 21 DPI due to blending of the corona of small lymphocytes with increased numbers of plasma cells and histiocytes in paracortical mantles (Fig. 3). Follicular centers contained individual necrotic lymphocytes and tingible-body macrophages probably representing apoptosis and leukophagia.[77] Medullary macrophages of 21 DPI lymph nodes often contained hemosiderin, and plasma cells were increased from the 14 DPI samples. The lymph node sections at 28 DPI were similar to 21 DPI, and had intrafollicular individual lymphocytolysis, sinus histiocytosis (increase in sinus macrophages), and increased plasma cells in medullary cords. In the lymph nodes from the 35 DPI dogs, a more distinct perifollicular mantle zone of small lymphocytes surrounded the germinal center, somewhat similar to the 7 DPI dogs. Germinal centers had individual necrotic lymphocytes and mitotic figures. Numerous plasma cells were present in the medulla of the 35 DPI lymph nodes.

Some lymph nodes contained sinuses filled by lacy

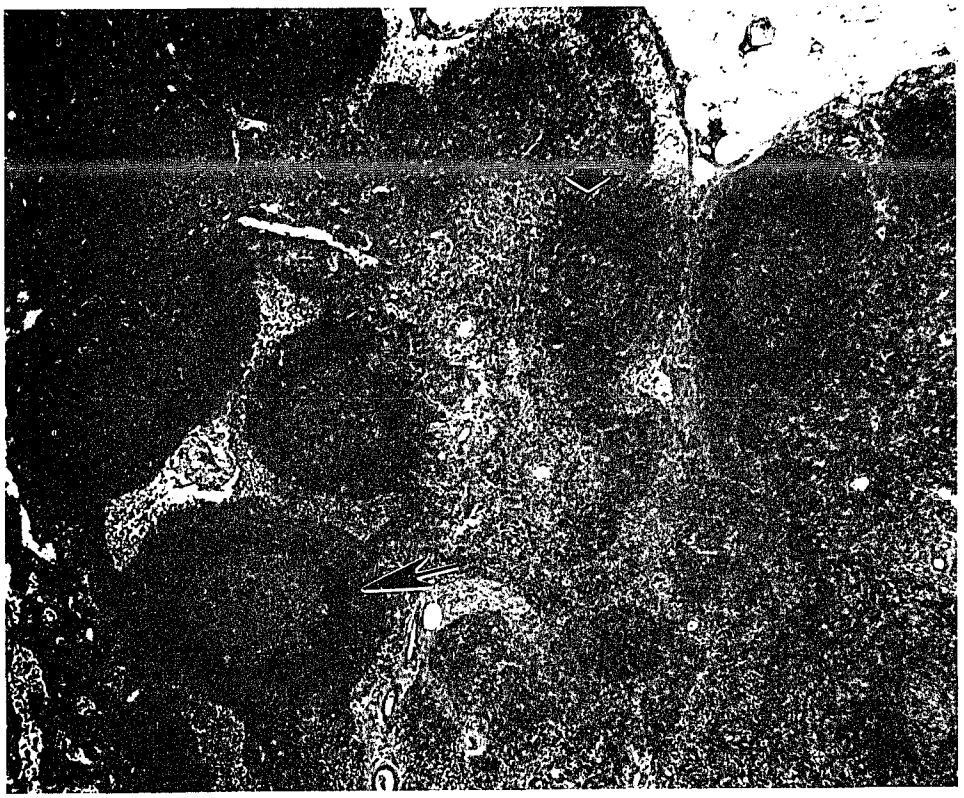

Fig. 1. Lymph node, E. platys infected dog, 14 DPI. Lymphoid hyperplasia with secondary follicles (arrow) consisting of germinal center surrounded by corona of darkly stained small lymphocytes. Note focal hemorrhage in corona of one paracortical follicle (broad arrowhead).

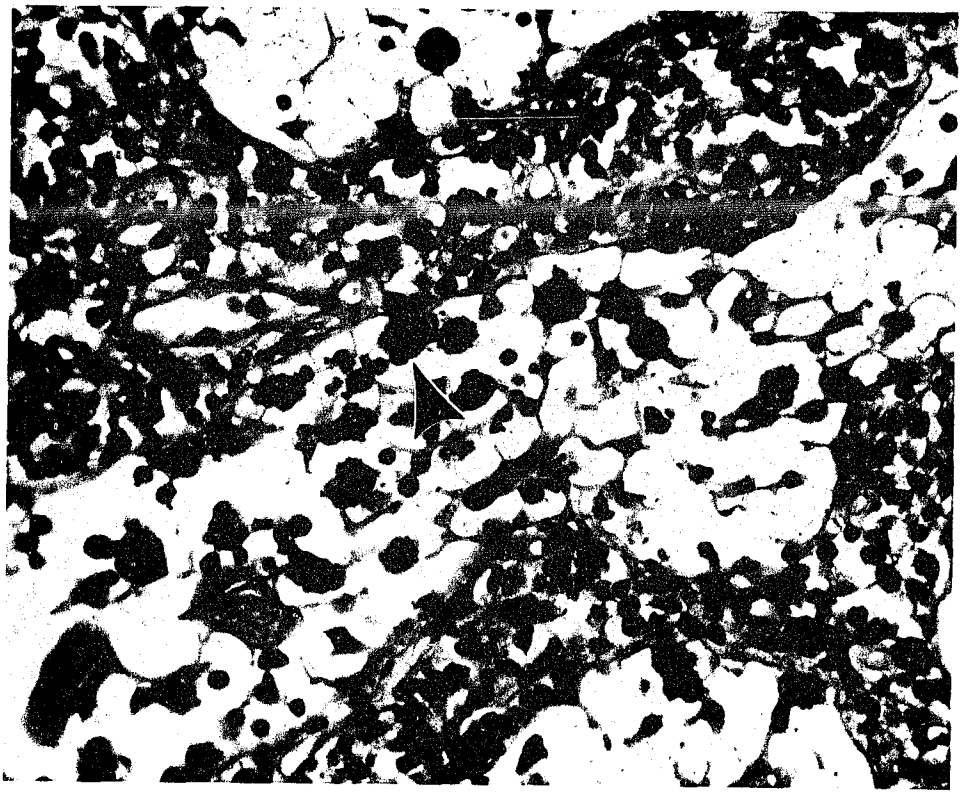

Fig. 2. Lymph node, E. platys infected dog, 14 DPI. Phagocytosis of erythrocytes by medullary sinusoidal macrophages (arrowhead).

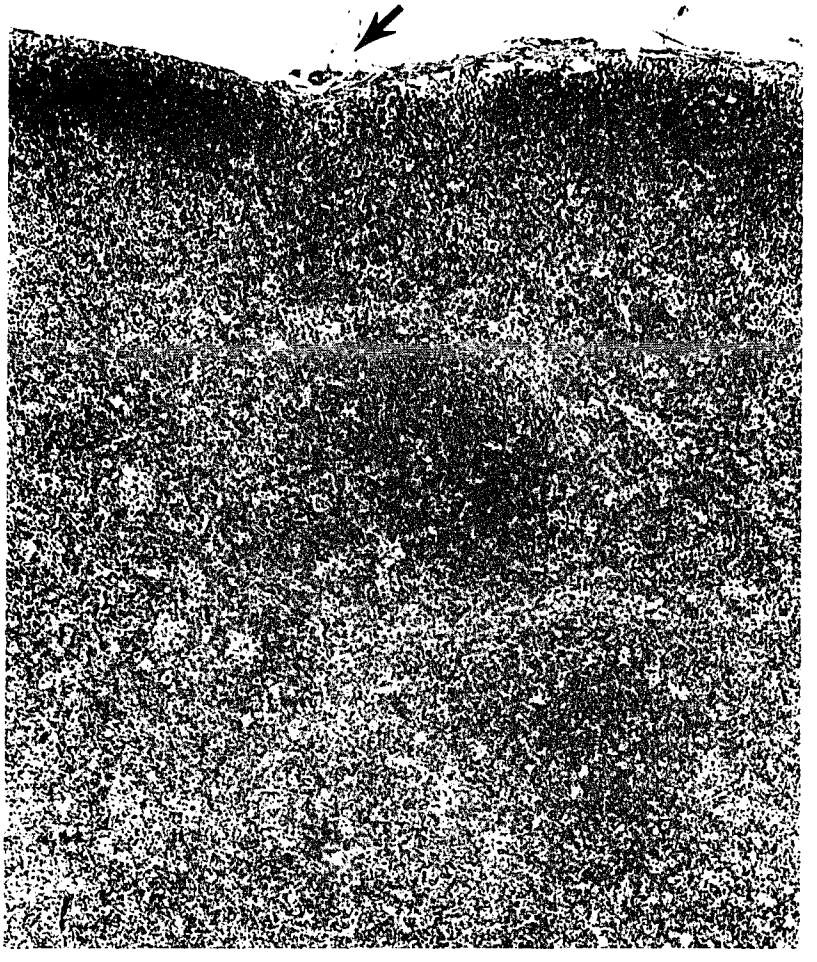

Fig. 3. Lymph node, E. platys infected dog, 21 DPI. Blending of the paracortex due to increase in plasma cells and histiocytes surrounding lymphoid follicles. Note lymphoid hyperplasia narrows subcapsular sinus and capsule is thin (arrow).

relatively hypocellular eosinophilic homogenous material on HE stain that was interpreted as edema. Occasionally, free and phagocytized erythrocytes were present in afferent capsular lymph vessels and subcapsular sinuses.

Hematopoietic cell hyperplasia occurred in proximal femoral bone marrow and spleen of all dogs (Fig. 4). Numerous megakaryocytes were observed in all samples from dogs each week, while increases of marrow megakaryocytes compared to the control dog were significant ($P<0.05$) in one dog examined on each 28 and 35 DPI. Abundant deposits of iron occurred in bone marrow from all dogs.

Lymphoid hyperplasia was evident in spleen. Splenic follicles had well developed germinal centers with numerous lymphoblasts. Individual lymphocyte necrosis and leukophagia occurred in follicles at the same interval as apoptosis in lymph nodes. Some follicles in all spleens except one of the 7 DPI dogs (mixed-bred) had crescent-shaped areas of hemorrhage located near the follicle periphery separating a portion of the mantle zone of small lymphocytes (Fig. 5). Intrafollicular hemorrhages were most numerous in the dogs examined 14 DPI. The hemorrhages persisted in infected dogs throughout the study and were less prominent in Beagle dogs than mixed-bred dogs.

There was nodular hyperplasia of splenic periarteriolar macrophage sheaths (PAMS or sheaths of

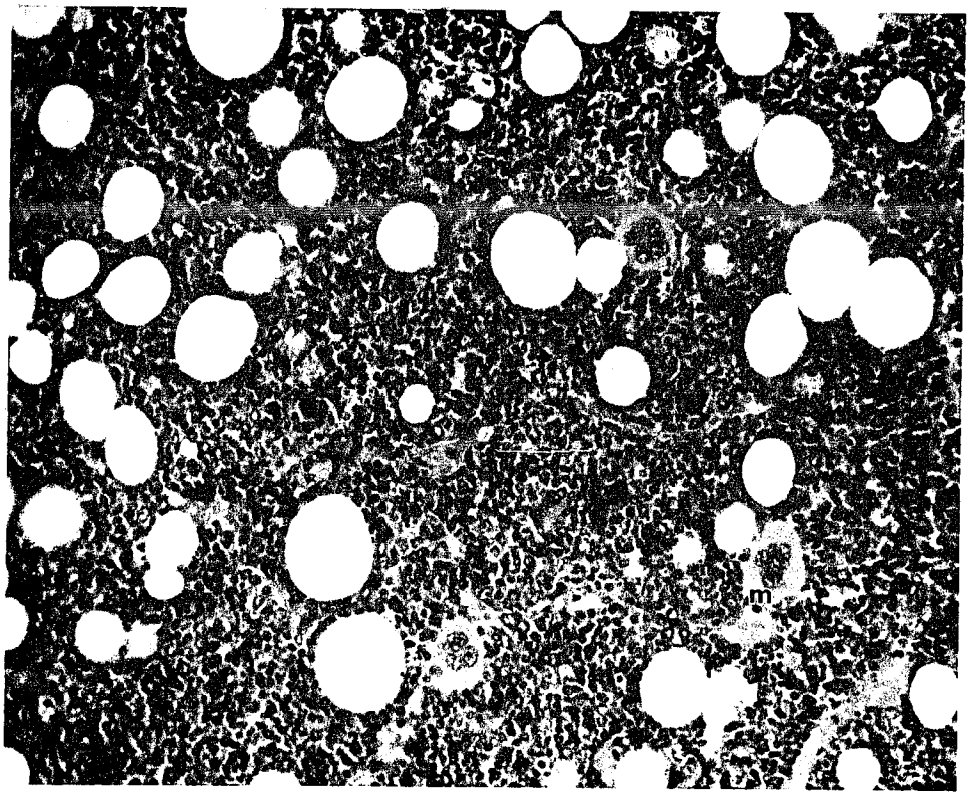

Fig. 4. Bone marrow, E. platys infected dog. Hematopoietic hyperplasia includes increased numbers of megakaryocytes (m).

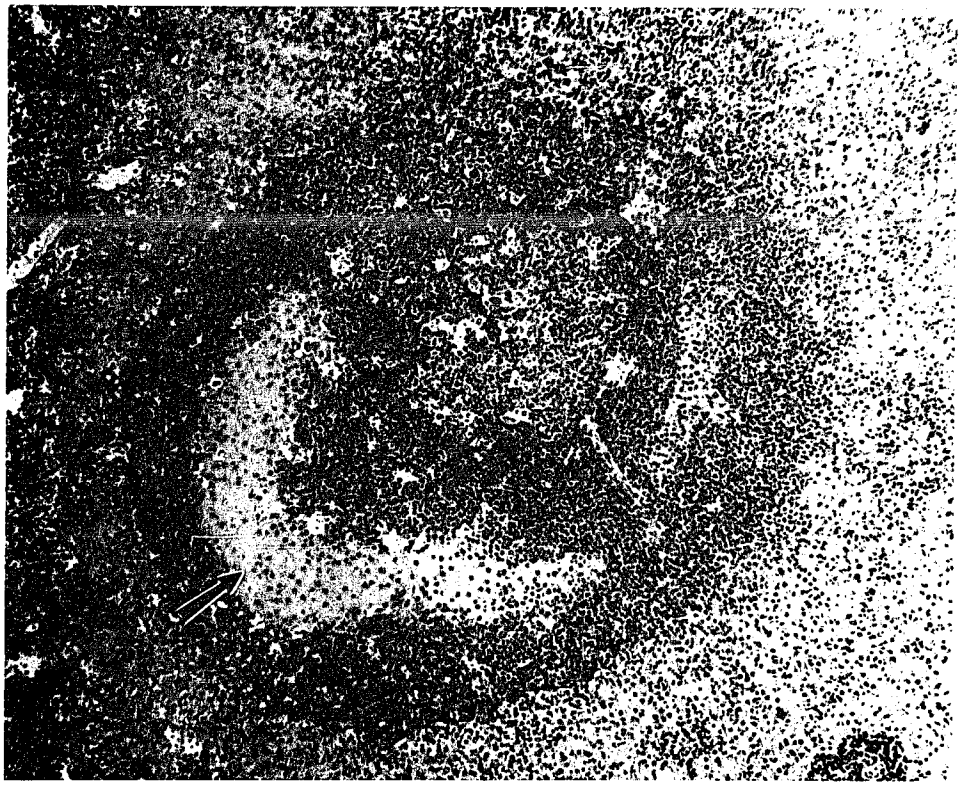

Fig. 5. White pulp, spleen, E. platys infected dog. Hyperplastic lymphoid follicle contains crescent-shaped intrafollicular hemorrhage (arrow).

Schweigger-Seidel).[78] These structures usually appeared to contain entrapped erythrocytes. Extramedullary hematopoiesis evidenced by increased nucleated erythrocytes and megakaryocytes in the red pulp, was most prominent on 28 DPI. Plasma cells became prominent in spleens examined beginning with the 14 DPI dogs. Erythrophagia and hemosiderin-laden macrophages were observed beginning in dogs examined 21 days PI. Reactive lymphoid hyperplasia was also observed in gut-associated lymphoid tissue.

Livers from all infected dogs had hepatocellular cytoplasmic vacuolation, multifocal hyperplastic nodules of Kupffer cells and portal lymphangiectasia. Sinusoids were mildly to moderately congested. Centrilobular hepatocytes were pale with multivesicular intracytoplasmic vacuolation (Fig. 6). Green-brown finely granular pigment interpreted to be bile pigment on Hall's stained sections, was present in the cytoplasm of centrilobular hepatocytes. Hepatocellular vacuolar change extended to include the periphery of the lobule (centriacinar) in some samples. Hepatocellular vacuoles contained PAS positive material, which was interpreted to be glycogen. There was multifocal replacement of few hepatocytes by small random aggregates of hyperplastic Kupffer cells that occasionally included a few lymphocytes and plasma cells (Fig. 6). Hepatic portal region lymphatics were characterized by

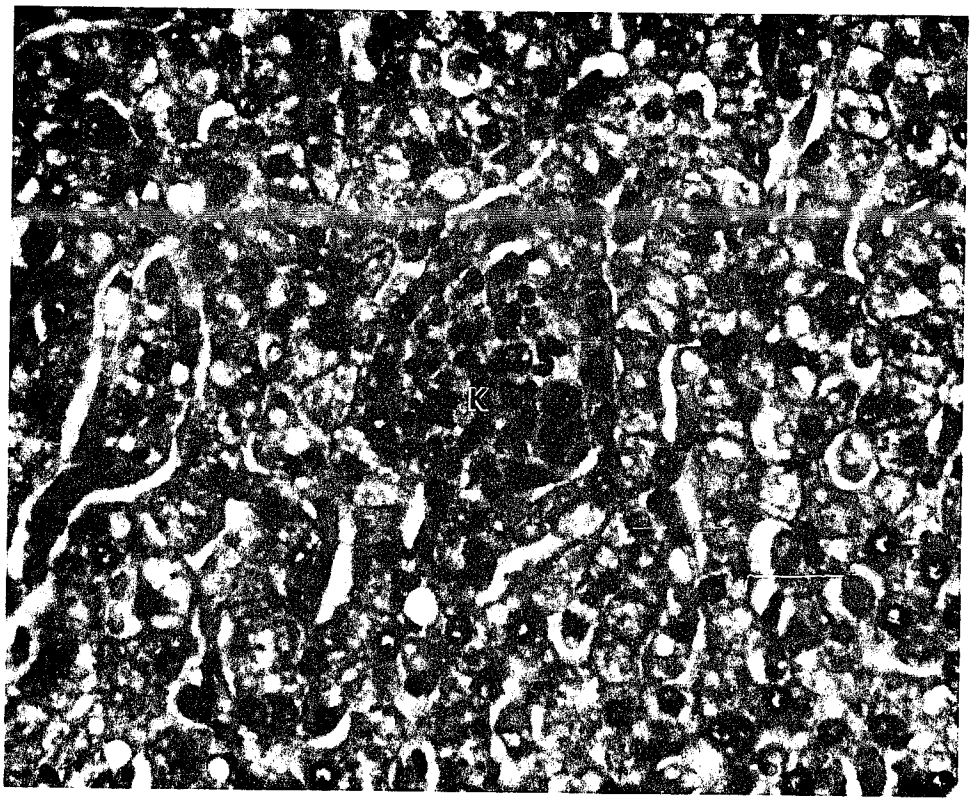

Fig. 6. Liver, E. platys infected dog. Hepatocytes contain multivesicular cytoplasmic vacuoles. Note focal replacement of hepatocytes by hyperplastic nodule of Kupffer cells (K).

increased luminal diameter that often contained homogenous eosinophilic acellular proteinaceous material (lymph) (Fig. 7). Portal lymphangiectasia was less prominent in dogs examined 28 and 35 DPI. At least one dog (21 DPI) also had multifocal expansion of spaces of Disse.

The kidneys contained very mild multifocal infiltrates of lymphocytes and plasma cells in the cortical interstitium, usually associated with cortical veins and renal corpusles. In both 7 DPI and one of the 14 DPI dogs, there was mild cortical lymphangiectasia. Cytoplasm of cortical tubules contained golden-green granular pigment in dogs examined from 7 to 28 DPI.

One of the infected dogs examined at 14 DPI, and both dogs examined at 21, 28, and 35 DPI, had prominent islands of thyroid gland parafollicular C-cells separating normal appearing thyroglobulin containing follicles. Some dogs (one dog each at 7, 14, and 21 DPI) also had prominent white nodular parathyroid glands (hyperplasia) larger than the control dog.

Scrotal skin from all but two dogs contained mild to moderate hyperplastic interface dermatitis. There was acanthosis with occasional focal exocytosis of lymphocytes or neutrophils amid epidermal epithelia. The superfical dermis was mildly to moderately diffusely infiltrated by lymphocytes and macrophages. Some dermal macrophages contained melanin pigment. In a few specimens there was

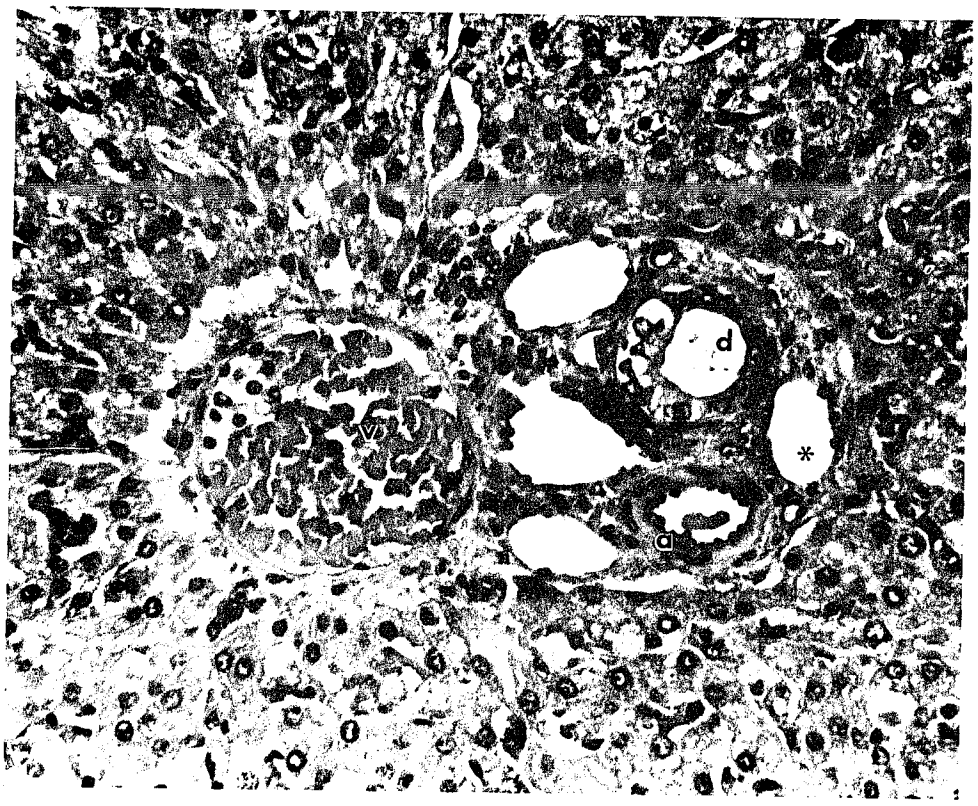

Fig. 7. Portal region (centriacinar), liver, E. platys infected dog. Four dilated lymphatics (one indicated by *) accompany bile ductule (d), hepatic arteriole (a), and portal vein (v).

focal erosion or ulceration with increased infiltration by neutrophils. No lesions indicative of histopathology were observed in dermal vasculature.

Platelets were not observed in paraffin sections of any tissues, except the eyes from all dogs. Platelets occurred individually and as small clumps in vascular lumens or in contact with choroid vessel endothelium (Fig. 8). Intrathrombocytic E. platys inclusions were not observed.

No lesions were observed by light microscopy of lung stained by HE, HBFP, or trichrome in any dogs (Fig 9).

Transmission electron microscopic examination of lung demonstrated pulmonary interalveolar septa thickened with irregular, amorphous, homogenous to mildly laminated medium electron dense deposits in all infected dogs (Figs 10, 11). Some deposits appeared to fill portions of the vascular lumen and be contiguous with basement membrane. In other foci, amorphous deposits appeared in the interstitium and were covered by endothelium. In dogs examined late in the study, these deposits occasionally had a faintly fibrillar character. Rare findings in lungs of individual dogs included focal intravascular platelet aggregates adhered to a naked basement membrane in one 14 DPI dog (Fig 12). Another dog (21 DPI) had a membrane-lined vacuole within a pulmonary microvascular endothelial cell that contained three spherical to ovoid

Fig 8. Choroid blood vessel, eye, E. platys infected dog. Platelet contact with endothelium (arrowheads). Note vessel lumen (l.), erythrocyte (r), and leukocytes (w).

Fig. 9. Lung, airway perfusion fixed specimen, E. platys infected dog. Interstitial thickening and cellular infiltrates were not observed.

Fig. 10. Interalveolar septum, lung, E. platys infected dog. Irregular amorphous to slightly laminated electron dense deposits (d). Note multifocal blending of deposit with microvascular basement membrane (arrow). Bar = 0.5 um.

Fig. 11. Microvasculature, interalveolar septum, lung, E. platys infected dog. An amorphous deposit (arrowhead) is contiguous with the basement membrane (b) and covered by endothelial cell (E). The endothelial cell nucleus (N) is included in the plane of section. An adjacent capillary lumen (c) lacks cells in plane of section and basement membrane (m) supports a second endothelial cell (E) and a type I alveolar pneumocyte (A). Bar = 0.2 um.

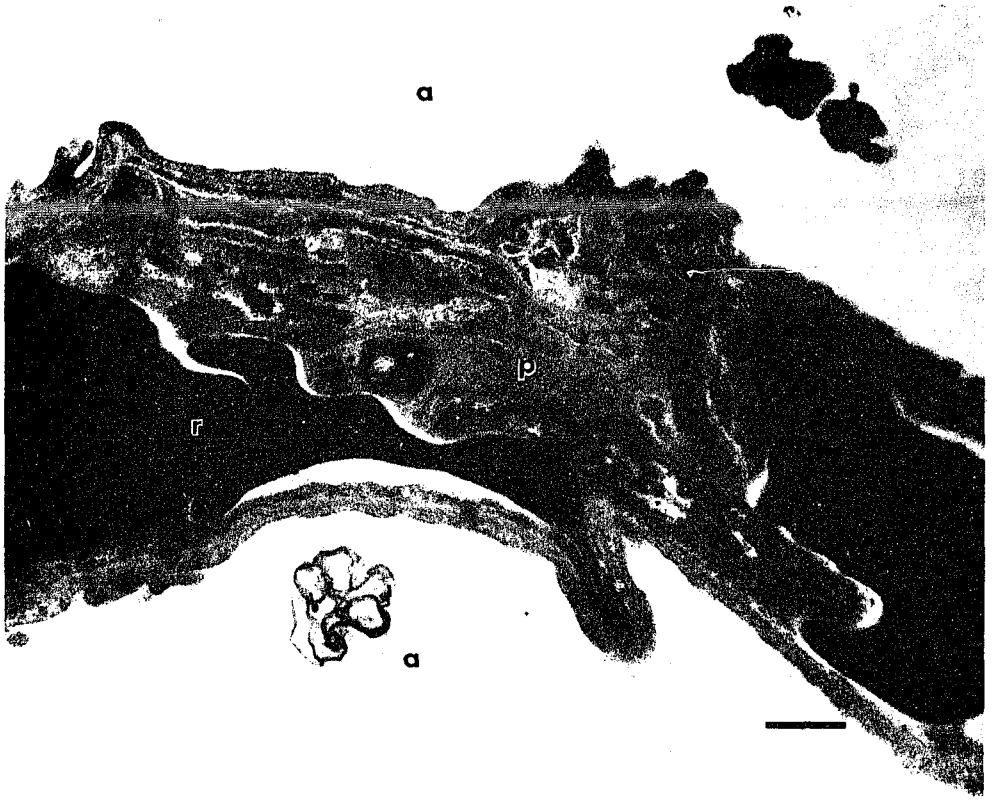

Fig. 12. Microvasculature, interalveolar septum, lung, E. platys infected dog, 14 DPI. Platelet aggregate (p) in lumen. Majority of platelets have few granules. Alveoli (a), erythrocyte (r) in vascular lumen. Bar = 0.5 um.

microorganisms with morphology consistent with ehrlichiae (Fig. 13). Ehrlichiae were 0.6-1.0 um diameter membrane bound structures with fibrillar and granular variably electron dense material (nucleoprotein). In another dog (7 DPI) there was focal pulmonary microvascular endothelial cell degeneration. The lumen of this vessel contained a 0.3 um diameter circumscribed structure with double unit membranes surrounding granular material resembling E. platys yet slightly smaller than reported dimensions.

Ultrastructural changes observed in other tissues of E. platys-infected dogs were not consistent. In sections of skin examined by TEM, a blood vessel contained a single focus of intravascular fibrin polymers and platelets in one dog examined 21 DPI (Fig. 14). Loss of discoid shape and pseudopod-like cytoplasmic extensions were observed in platelets containing few granules. Evidence of leukocyte degranulation was also observed. The endothelium of the vessel within the visible transverse section was intact.

Transmission electron microscopy of spleen from a dog examined 14 DPI revealed two spherical to ovoid approximately 1 um diameter structures morphologically similar to E. platys (Figs. 15, 16). Organisms were observed within a membrane lined vacuole within a degenerate splenic cell. The identity of the type of cell containing the organisms was not apparent morphologically

Fig. 13. Microvasculature, interalveolar septum, lung, E. platys infected dog 21 DPI. Endothelial cell (E) cytoplasmic vacuole (v) contains structures that may represent ehrlichial organisms. Cleaved appearing structure (arrowhead) may represent organism replication by binary fission. The capillary lumen is labeled (c). Bar = 0.25 um.

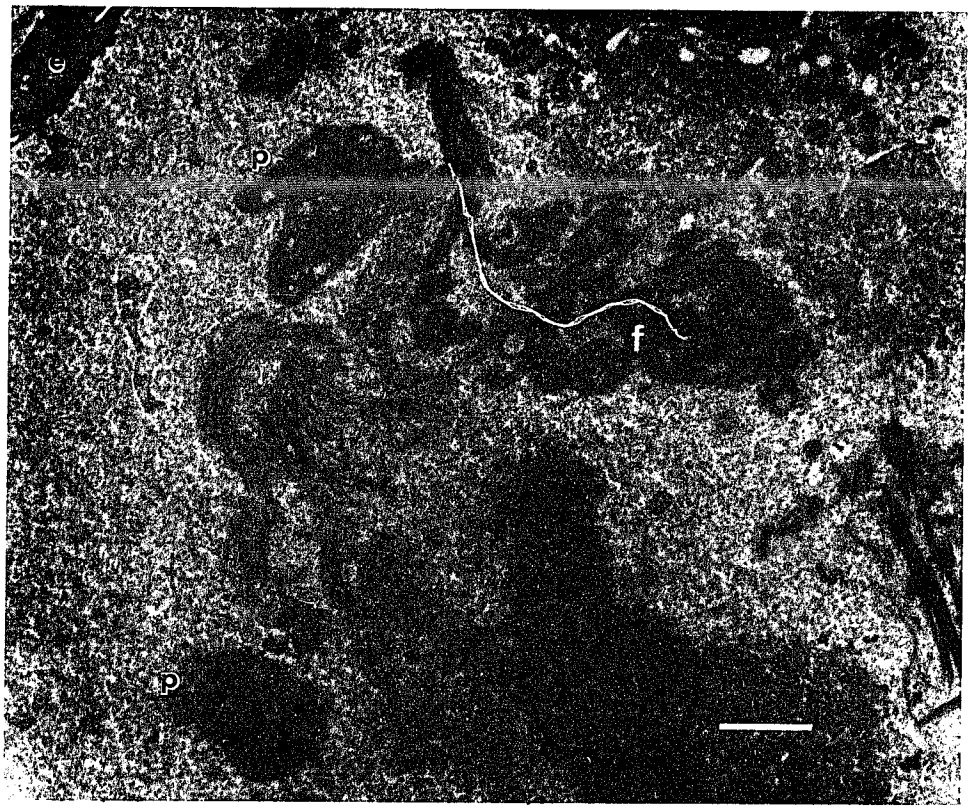

Fig. 14. Blood vessel lumen, skin, E. platys infected dog, 21 DPI. Note intravascular fibrin (f) and platelets (p), some with pseudopodia. Vessel endothelial lining in upper left of figure (e). Bar = 1.0 um.

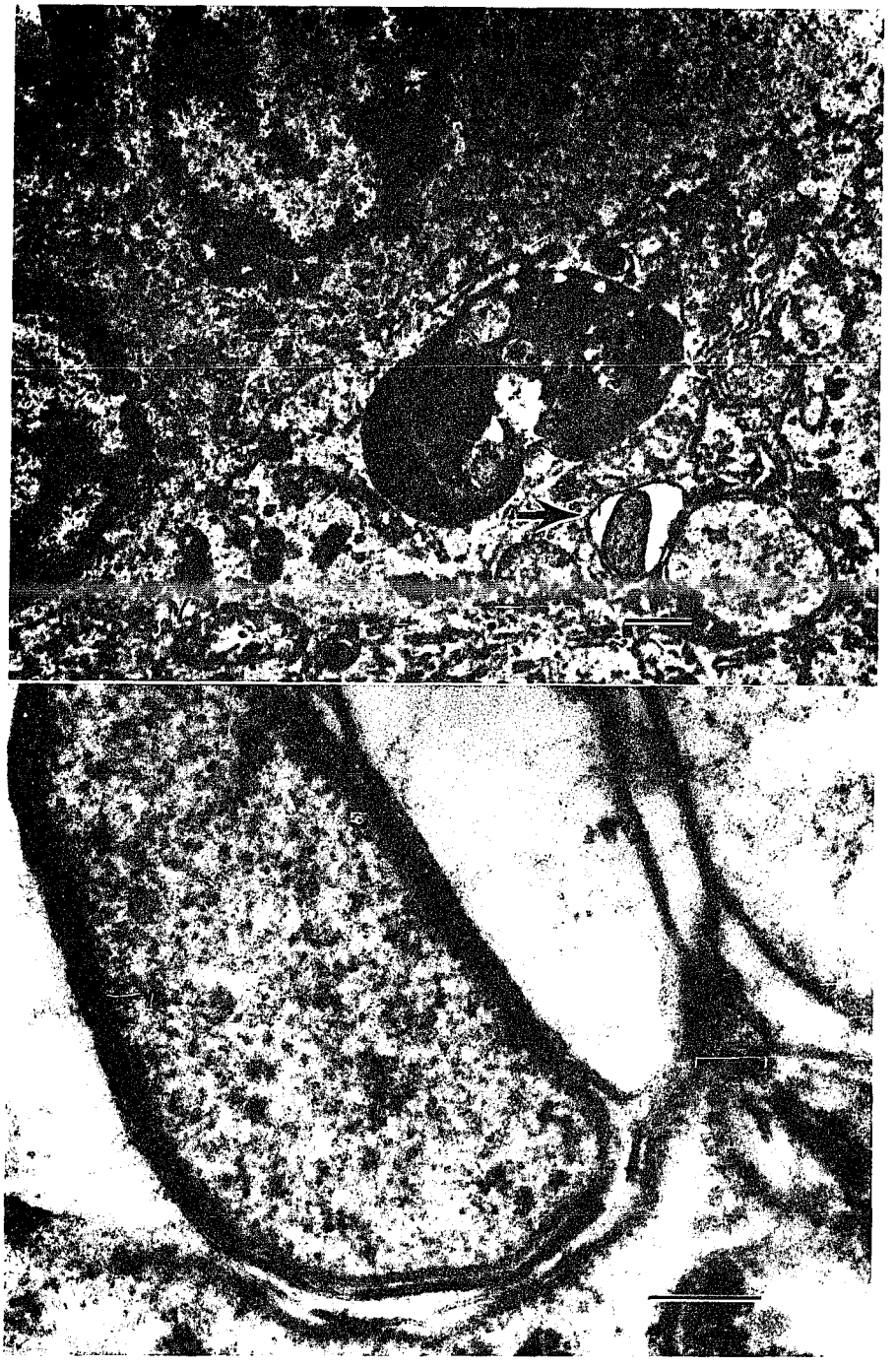

Fig. 15. Red pulp, spleen, E. platys infected dog, 14 DPI. Structure resembling ehrlichia (arrowhead) in cytoplasmic vacuole of unidentified spleen cell. Bar = 0.5 um.

Fig. 16. Red pulp, spleen, E. platys infected dog, 14 DPI. Detail of ehrlichia in Fig. 15 spleen cell vacuole. Bar = 0.1 um.

because cytolysis obscured cytoplasmic ultrastructure. Ovoid structures resembling swollen mitochondria and an irregular electron dense mass probably representing nuclear pyknosis were observed in addition to E. platys inclusions. Organisms were not observed in other tissue sections examined stained either with HE, Machiavello, or modified Steiner silver stains. None of the lesions observed in E. platys-infected dogs was observed in the control dog tissues.

DISCUSSION

All experimentally inoculated dogs became infected and developed lesions attributable to acute E. platys infection. Clinical condition, hemograms, and serum antibody responses were similar to previous reports.[7,12,17] Many of the lesions of acute E. platys infection documented here were similar to acute E. canis infection.[25] Proliferation of lymphocytes and macrophages with mild to marked plasmacytosis in lymph nodes, spleen, liver and kidney indicated systemic antigenic stimulation by E. platys. In addition, the lymphoid tissue hyperplasia observed was similar to canine neorickettsiosis.[79] While episodes of thrombocytopenia were followed by approximate return of platelet concentration to preinfection levels in dogs surviving longer than 21 DPI, morphologic lesions and serum antibody response was not cyclic.

The hematopoietic hyperplasia that occurred in bone marrow and spleen was similar to what is seen in dogs with acute E. canis infection.[25] The thrombocytopenia induced by E. platys was accompanied by increased megakaryocytopoiesis in all infected dogs, which were statistically siginificant at 28 and 35 DPI. Recovery from marked thrombocytopenia in E. platys infection was accompanied by the appearance of megaplatelets (large, shift, or stress platelets) in peripheral blood (unpublished observation). These findings indicate that E. platys causes a regenerative thrombocytopenia, and was not caused by lack of platelet production.[24] Ehrlichia platys inclusions were not observed in megakaryocytes. Cyclic change in megakaryocyte numbers was not observed, indicating that immune-mediated cyclic interruption in megakaryocytopoiesis does not occur in E. platys infection. Such a change in megakaryocytes has been documented in thrombocytopenic puppies inoculated intraperitoneally with anti-canine platelet serum.[80] Once per week sampling intervals in this study may have precluded observation of some changes in megakaryocytes. What role, if any, abnormal platelet release from megakaryocytes in marrow and extramedullary sites plays in the episodic nature of ICT is undetermined.

Interstitial pneumonia in E. platys infection was the only previously reported morphologic lesion.[7,36]

Interstitial pneumonia in dogs infected with E. platys for 60 days was charcterized by fibrosis of interalveolar septa.[7] In another previous study of acute E. platys infection, thickened interalveolar septa were described.[35] Lungs from acutely infected dogs examined in the present study were not pneumonic nor fibrotic. Differences in pulmonary findings from previous studies[7,36] may reflect differences in tissue processing, or E. platys strain or inoculum differences. In previous studies of E. platys[7,36] and E. canis[13,17,25,27] infections, lungs were presumably fixed by routine immersion in fixative. Lungs fixed by immersion alone can be difficult to interpret following tissue collapse due to elastic recoil inherent in lung. Artefactual thickening of interalveolar septa is prevented by the pulmonary airway perfusion fixation, as used in this investigation, and may explain results divergent from those previously reported.[7,36]

The nature of the amorphous homogeneous laminated thickenings in alveolar septal basement membranes, that appeared slightly fibrillar in electron micrographs from dogs sampled late in the study, was not determined. The amorphous material had an electron density somewhat similar to basal lamina, and may represent insudation of plasma protein, deposition of previously formed antigen-antibody complexes and possibly complement, deposition of rickettsial antigen, or deposition of fibrin,[81] or may

even represent an artefact of lethal injection. Formation of these thickenings may follow endothelial injury as in diabetic microangiopathy or result from transendothelial transport or diffusion of inflammatory mediators. Such deposits have not been described in other reports of ehrlichiosis. Accumulation of such material at the basal laminae may serve to stimulate subsequent fibrosis that would be apparent only in dogs surviving longer than the dogs in the present study (35 DPI).

There was rare ultrastructural evidence of intravascular coagulation and platelet aggregation in lungs and dermal vessels examined. The infrequent morphologic evidence of intravascular coagulation may be due to the low frequency with which such coagulation may have occurred, an acquired platelet functional defect possibly involving complement depletion similar to E. canis infection,[23] or active fibrinolysis.[82] Parameters used to assess coagulation such as fibrin degradation products were not measured.

In addition to hyperplasia of both splenic lymphoid follicles and PAMS in all E. platys infected dogs, which paralleled the changes in lymph nodes, intrafollicular hemorrhage occurred in the spleens of all dogs except one at 7 DPI. Intrafollicular hemorrhage appeared as crescent-shaped lesions in tissue sections, and was present in perfusion-fixed as well as immersion-fixed

samples. Splenic intrafollicular hemorrhages have been reported in experimental simian hemorrhagic fever virus (SHFV), immune mediated thrombocytopenia, hyperparathyroidism, neorickettsiosis, and E. canis. Some splenic follicular hemorrhages in E. platys-infected dogs were focal or concentric. Marked concentric splenic follicular hemorrhage occurs in primates in extremis or dead from SHFV.[83] Simian hemorrhagic fever virus induced splenic follicular hemorrhage appeared to be associated with disseminated intravascular coagulation (DIC). The occurrence of canine splenic follicular hemorrhage in experimental thrombocytopenic purpura induced by administration of antiplatelet serum,[80] suggests that immune mediated platelet removal may be associated with splenic follicular hemorrhage. Following administration of antiplatelet serum, however, splenic follicular hemorrhage appeared in dogs 12-13 DPI when blood platelet numbers had returned to normal levels following thrombocytopenia. Splenic follicular hemorrhage has been observed in people with chronic clinical thrombopenic purpura.[80] The pathogenesis of splenic follicular hemorrhage was not determined from these earlier studies.

The finding of thyroid gland parafollicular C-cell hyperplasia in a number of dogs acutely infected with E. platys was noted here since splenic follicular hemorrhage was observed in dogs injected with parathyroid gland

extract.[84] While increased endocrine gland function does not always correlate with hyperplasia, hormones may influence formation of splenic follicular hemorrhage and additional study is needed. Enlargement of the parathyroid gland may also be an incidental peculiarity of the laboratory Beagle dog.

Focal intrafollicular splenic hemorrhage associated with coalescent foci of lymphoid necrosis was also described in splenic follicles of dogs infected with Neorickettsia helminthoeca.[79,85] These lesions appeared to be different from the splenic follicular hemorrhage of ehrlichiae infected dogs.[25]

The mechanism and significance of splenic follicular hemorrhage in Ehrlichia spp. infected dogs is unknown. The splenic lesions observed in the E. platys infected dogs in this study were similar to those reported in dogs with acute E. canis infection.[25] In that report,[25] splenic follicular hemorrhages were thought to be a site of platelet pooling contributing to thrombocytopenia. The appearance of splenic follicular hemorrhage in E. platys infected dogs corresponded to the onset of thrombocytopenia. Splenic follicular hemorrhages were variable, but persisted in all spleens unrelated to the cycling of blood platelets. Splenic follicular hemorrhages may be a site of platelet sequestration during acute E. platys infection, since regeneration of blood

platelet concentration, following platelet nadir, does not reach full preinfection levels. While splenic sequestration of platelets may well occur, the mechanism may be more likely related to the effects of ehrlichiae on a blood-spleen barrier controlled by complex red pulp reticular stromal cell function rather that platelet pooling in white pulp follicular hemorrhages. Splenic reticular cells, that control blood flow through filtration locules, can become activated in response to certain antigens.[86] In mice infected with murine malaria (Plasmodium yoelii), these specialized stromal cells secrete protein, branch to form cytoplasmic processes, and undergo mitosis forming a blood-spleen barrier that protects splenic hematopoiesis from the parasite. Such "protected" myeloid elements are released into circulation following an episode of rapid immune-mediated clearance of malaria-parasitized erythrocytes. Speculatively, such mechanisms could partially explain the cyclic nature of thrombocytopenia in E. platys infection. Additional study is needed to determine the potential role of the spleen in controlling cycles of thrombocytopenia in E. platys infection.

Hyperplastic splenic PAMS contained entrapped erythrocytes. Phagocytosis of erythrocytes and deposits of hemosiderin in lymph node, spleen, and bone marrow macrophages of dogs examined after 14 DPI indicated

increased erythrocyte destruction. This is probably related to the occurence of anemia of inflammation that occurs in E. platys-infected dogs.[31]

Electron microscopic examination of splenic red pulp revealed rare E. platys organisms. E. platys subunits (inclusions) were observed in a membrane lined cytoplasmic vacuole of a spleen cell undergoing plasmacytolysis. Definitive identification of the cell type could not be made, but probably represented either direct infection or phagocytosis of an infected cell or organism by a splenic macrophage. Direct immunofluorescent antibody localization of E. platys antigen in spleen, liver, bone marrow, lymph node mononuclear cells in the 14 day dogs coincided with initial thrombocytopenia. Thus in addition to erythrocyte trapping, resident tissue macrophages may serve as sites of increased platelet removal in ICT as in E. canis infection.[22,24]

Transient increases in serum levels of ALT and ALP corresponded to the appearance of hepatocellular vacuolation, cholestasis, portal lymphangiectasia, and mild proliferation of Kupffer cells in livers of E. platys infected dogs. Lymphangiectasia and Kupffer cell hyperplasia were observed in the majority of dogs, while hepatocellular changes were variable. The onset of serum enzyme activity and hepatopathy was temporally related, and probably reflected the onset of cholestasis and

hepatocellular damage.[87] Similar events have also been observed in acute E. canis infection.[25] Kupffer cell hyperplasia seen in E. platys infection is involved in host response to rickettsial disease,[88] and was similarly reported in both neorickettsiosis,[79] and acute E. canis infection.[25] Kupffer cell hyperplasia displaced a few hepatocytes and may contribute to elevation of serum enzymes.[25] Kupffer nodules tended to increase mildly in dogs sampled late in the study at 28 and 35 DPI, however, when only one dog had increased ALT or ALP activity.

Hepatic portal lymphangiectasia, mild renal lymphangiectasia, and lymph node edema may reflect formation and accumulation of "organ" edema. Clinically evident extracellular compartment fluid accumulation in connective tissue spaces that occurs in horses with E. equi infection,[89] and some dogs with E. canis infection[16] was not observed in E. platys-infected dogs. The mechanism of mild edema formation in several parenchymatous organs in E. platys infection is unknown. While it is probable that acute phase phenomena affecting the liver, accompanied by juxtaparenchymal vascular injury, may influence local organ edema formation,[34] in acute E. platys infection; some degree of congestive cardiovascular disease as a cause can not be eliminated.

Cardiogenic edema must also be considered as a possible cause of hepatic edema in E. platys-infected dogs

in view of hepatic sinusoidal congestion. Acute passive congestion can result in uniform congestion of hepatic sinusoids and portal lymphangiectasia.[90] In this study however, sinusoidal congestion was considered peracute and incidental to heart failure induced by euthanasia. Neither signs of congestive heart failure nor presence of significant hepatic fibrosis is present in any of the dogs in this study. Further, portal or renal lymphangiectasia are not associated with peracute heart failure of euthanasia. Increased capillary pressure occurs in cardiac failure which generally results in dependent edema.[91] Dependent edema did not occur in acute E. platys infection. Hepatic portal lymphagiectasia is not generally associated with the increased blood volume of chronic passive congestion that occurs in dogs with congestive heart failure.

Hepatic edema is reported to occur in up to 55% of human cases of thrombopenic purpura,[80] which indicates an immune mediated component may be involved in the mild "organ" edema observed in E. platys infected dogs.

Altered liver biochemistry during acute E. platys infection, was expressed by changes in plasma protein constituents,[31] as well as increases in serum ALT and ALP. Plasma increases in alpha and gamma globulins and a decrease in albumin,[31] may have reflected acute phase of inflammation phenomena,[34] and may also contribute to the

development of edema.

Platelet-endothelium tropism is necessary for normal vascular integrity.[92] Morphologic evidence of intravascular coagulation in pulmonary and dermal vessels coupled with the possiblity of endothelial infection in acute ICT, makes vascular compromise a distinct possibility. Vascular injury alters permeability and favors transudation of plasma protein contributing to edema formation.[91] Direct endothelial cell infection is not necessary, for modifications in endothelial cell surface structure alone can produce a loss of thromboresistance or increased vascular permeability.[29]

Except in platelets in peripheral blood smears, *E. platys* organisms were observed in tissues only rarely. TEM revealed a structure similar to *E. platys* in the lumen of pulmonary microvasculature at 7 DPI. *Ehrlichia platys* subunits were also observed in the spleen at 14 DPI, and pulmonary microvascular endothelium at 21 DPI. Vascular lesions were subtle and vasculitis similar to either acute *E. canis*[25] or *R. rickettsii* infection,[76] was not observed in any tissues from *E. platys* infected dogs. Platelet clumps were in contact with choroid vessel endothelium in paraffin sections of eyes. Platelets are not routinely observed in paraffin embedded tissue sections for a variety of possible reasons. Preservation of platelets in tissue sections requires special

methods,[80] and the appearance of platelets in eyes of E. platys infected dogs may reflect the fixation method or rapidity with which eyes were preferentially placed in fixative. In routinely processed tissues platelets may be confused with precipitated plasma proteins, or blood cell detritis.[80] Platelet clumping and endothelial contact may be evidence of platelet adhesion and aggregation. Evidence of platelet adhesion and degranulation in one dog was observed by TEM in lung and skin. Whether similar events occurred in eyes or other organs of E. platys-infected dogs is unknown.

Scrotal dermatitis that occurred in these dogs probably resulted from environmental conditions in the kennel and was considered incidental.

Serum antibody response in E. platys-infected dogs was similar to a previous report of ICT.[12] Serum antibody titers to E. platys progressively increased in six of seven dogs. Two of ten dogs necropsied early in the study did not have demonstrable titers, but were thrombocytopenic. The relationship of host antibody response to development of thrombocytopenia is unknown.

Acute E. platys infection caused increased megakaryocytopoiesis in bone marrow and spleen and hyperplasia of lymphoid elements in spleen and lymph nodes similar to acute E. canis infection.[25] Following a 10-14 day incubation period, E. platys caused marked

thrombocytopenia and minimal tissue hemorrhage and edema, which could result from endothelium injury, platelet consumption by intravascular coagulation, or platelet removal by proliferative resident tissue macrophages. Thrombocytopenia was followed by rapid recovery of blood platelet concentration at 15-18 days post inoculation.

Both E. platys and E. canis cause similar anatomic lesions in acute experimental infection. Distinction between these two ehrlichiae on a purely morphologic basis would be difficult in infected dogs. Interstitial pneumonia and vasculitis reported to occur in E. canis infection did not occur in the E. platys infected dogs on this study. The mechanism of thrombocytopenia in E. platys infection would appear to be complex and might be associated with removal of platelets by resident tissue macrophages and, to a lesser degree, consumptive coagulopathy.

TABLE 1. Blood platelet concentrations and parasitemia in dogs infected with Ehrlichia platys

		Blood Platelet Concentration ($X 10^3$/ul)		Platelet Parasitemia (%)	
DPI	N	MEAN	S.D.	MEAN	S.D.
0	10	293	50	0	0
3	10	286	61	0	0
7	10	217	52	0	0
10	8	148*	92	3.9	2.6
14	8	44*	18	4.9	6.1
17	6	36*	26	0.4	0.4
21	6	46*	34	0	0
24	4	152*	135	0.2	0.1
28	4	93*	58	0.3	0.4

DPI = days post infection
N = number of dogs sampled
* = change is significant ($P<0.05$) from day 0.

CHAPTER III

Evaluation of The Brown Dog Tick, Rhipicephalus sanguineus (Acari: Ixodidae), as a Potential Vector of Ehrlichia platys

ABSTRACT

Rhipicephalus sanguineus (Acari: Ixodidae) transmits several diseases among dogs including Ehrlichia canis infection. The role of Rhipicephalus sanguineus as a biologic vector for Ehrlichia platys, the rickettsial agent of infectious canine cyclic thrombocytopenia, was studied in dogs. Laboratory cultured, pathogen-free nymph ticks were fed to repletion on dogs acutely infected with Ehrlichia platys. Tick engorgement coincided with the development of initial parasitemia and thrombocytopenia in the infected dogs. Following repletion, nymph ticks were allowed to molt under controlled conditions. One month-old unfed E. platys-exposed adult ticks failed to infect naive dogs in animal transmission studies. The presence of Ehrlichia platys was not detected in midgut or salivary gland of similarly exposed ticks by light and transmission electron microscopy. Frozen sections of E. platys-exposed tick midgut and salivary gland were stained by immunocytochemical technique; the presence of Ehrlichia

platys antigen in these tissues was not detected. These studies indicate that Rhipicephalus sanguineus may not transmit Ehrlichia platys infection.

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INTRODUCTION

Members of the family Rickettsiaceae are adapted to survival in an arthropod host, and ixodid (hard) ticks are the most common vectors of rickettsiae. Infection of vertebrates is not essential to the survival of rickettsiae, and ixodid ticks serve to maintain rickettsiae in nature. Rickettsiae infect vertebrates, when their arthropod host avails itself of a meal of blood.[2] Three ehrlichial agents have known ixodid tick vectors. Ixodes ricinus transmits Ehrlichia phagocytophila among cattle in Europe.[1] Hyalomma sp. transmits Ehrlichia bovis in the Middle East, North and Central Africa, and Sri Lanka.[38] The brown dog tick, Rhipicephalus sanguineus, was incriminated as the vector of Ehrlichia canis (E. canis) by Donatien and Lestoquard in their original description in 1935 of canine ehrlichiosis.[16,27] Infection, reproduction, and dissemination of E. canis within Rhipicephalus sanguineus

(R. sanguineus) followed by subsequent rickettsial transmission has been demonstrated using ticks fed on experimentally infected dogs.[9,41,42] The world-wide temperate to tropical range of canine ehrlichiosis is thought to be limited only by the geographic niche of R. sanguineus.[93] Recently, arthropod transmission for other members of ehrlichieae has been investigated including human sennetsu ehrlichiosis (E. sennetsu)[53] and Potomac horse fever (E. risticii).[52]

Ehrlichia platys (E. platys), the agent of infectious canine cyclic thrombocytopenia (ICT), is morphologically similar to E. canis, but parasitizes circulating canine platelets rather than mononuclear leukocytes. E. platys infection in dogs results in intrathrombocytic parasitemia and subsequent cycles of thrombocytopenia that recur at 7-14 day intervals. Remission of E. platys infection appears to be spontaneous.[7,12] Methods of E. platys transmission between dogs and how the microorganism persists in nature is unknown,[7] but R. saguineus has been considered to be the biologic vector of E. platys.[94]

Arthropod-borne diseases are typically seasonal and noncontagious.[52] Because of the predominantly occult clinical manifestations of ICT, the incidence and possible seasonal occurrence are not known.[7,12]

Serological surveys by immunofluorescent antibody

(IFA) assay have documented canine exposure to E. platys in several states in North America.[12,94] In one survey 9 of 26 thrombocytopenic dogs (35%) had serum antibodies to E. platys. The majority of these dogs also tested positive for serum antibodies to E. canis, which do not cross react with E. platys antigens.[12] In a study of Louisiana dogs, 11 of 27 ill thrombocytopenic dogs (40.7%) had a serum antibody titer to E. platys and 32 of 59 clinically healthy kenneled dogs (50.2%) also had positive titer to E. platys antigen.[94] Anti-E. platys antibody was detected in a group of humans that also had anti-E. canis antibodies related to recently reported human ehrlichia exposure and history of tick bite[11,95] (French TW, personal communication, ACVP meeting, Montery, CA, 1987; Fishbein DB, CDC, Atlanta, GA, personal communication, 1988). The possibility of concurrent infection in dogs by E. canis and E. platys has led to the postulation of a common biologic vector.[16,94] Therefore, R. sanguineus was evaluated as a potential biologic vector of E. platys. In addition, this study was designed to identify sites of ehrlichial infection and replication in the tick using transmission electron microscopy and immunocytochemistry.

MATERIALS AND METHODS

Experimental Design. Dogs used in experimental E.

platys infection were inoculated intravenously with blood from a dog acutely infected with E. platys. A dog inoculated at the same time with blood from an uninfected dog served as control. Laboratory raised pathogen-free nymph R. sanguineus ticks were confined in tick feeding capsules on all dogs and allowed to attach to dogs and engorge with blood. Tick placement in capsules was staggered beginning from 100 to 150 hours post E. platys inoculation in order to extend tick exposure to infected dogs and coordinate tick engorgement with the development of initial parasitemia and thrombocytopenia in the infected dogs. Following repletion with blood, nymph ticks were allowed to molt under controlled conditions in an acaridarium.

A separate group of dogs were used as uninfected naive dogs considered susceptible to E. platys, for feeding the molted adult ticks to assess their ability to transmit E. platys. One month-old unfed adult ticks that had fed on E. platys-infected dogs as nymphs were infested on the naive dogs. Molted adult ticks fed on the uninfected control dog as nymphs, were allowed to attach to another naive dog as control. Dogs were monitored for signs of E. platys infection by platelet concentration, blood smear examination, and E. platys serum antibody determination for 60 days post tick infestation. Serum

was collected for E. platys antibody determination by immunofluorescent antibody assay (Appendix A) from the naive tick infested dogs on 12, 22, 30, and 55 days post infestation. Following the 60 day observation period, dogs were challenged by intravenous inoculation of E. platys platelet-rich plasma. Additional one month-old unfed adult ticks fed as nymphs on the same E. platys-infected dogs and control dog described above, were dissected and samples of midgut and salivary gland were examined for evidence of E. platys organisms by light and transmission electron microscopy and immunocytochemistry.

Dogs. Six healthy conditioned purebred Beagle dogs with no detectable serum antibody titers to either E. platys or E. canis and platelet concentrations within laboratory reference ranges were used in the animal transmission study. None of the dogs had any known previous exposure to ticks. Dogs were housed within the ectoparasite-free confines of the Louisiana State University animal vivarium administered according to the American Association for Accreditation of Laboratory Animal Care. Two dogs were infected with E. platys and used to expose nymph ticks to E. platys following voluntary attachment to skin and engorgement with blood. Another two dogs remained uninfected and were used as naive susceptible dogs to feed the adult ticks that had

molted from E. platys-exposed nymphs. The E. platys-infected dogs and the naive susceptible dogs each were matched with one uninfected dog that served as a control.

E. platys inoculum. Two dogs were inoculated intravenously with 20 ml heparinized blood with 50.0 X 10^3 platelets/ul collected from a dog with acute E. platys infection during initial appearance of infected platelets in blood. Approximately 5% of the platelets had inclusions or morulae of E. platys. This acutely infected dog serving as source of inoculum for this experiment had been inoculated with a frozen stabilite of E. platys platelet-rich plasma also with 5% of platelets containing E. platys inclusions. Platelet stabilite containing ampules stored in liquid nitrogen were thawed, and centrifuged at 10,000 g, and resuspended in original volume of sterile physiologic saline to wash platelets of 10% dimethyl sulfoxide (DMSO) used as cryoprotectant. The control dog that was matched with the dogs experimentally infected with E. platys was inoculated intravenously at the same time with 20 ml of fresh heparinized blood from a healthy uninfected dog.

Experimental E. platys infection. E. platys infected dogs and control were inoculated with blood (0 days post inoculation [0 DPI]). Dogs were monitored daily by blood platelet concentration, and examination of blood smears

for E. platys inclusions or morulae in platelets. Platelets in ethylenediaminetetraacetic acid anticoagulated blood were counted using 1:1000 dilutions in ammonium oxalate solution and a hemacytometer (Unopette^{tm}, 5855, Becton Dickinson, Rutherford, NJ). Serum from experimentally inoculated dogs was collected for determination of antibody titers to E. platys and E. canis on 22 DPI and stored frozen until analyzed by immunofluorescent antibody asssay (Appendix A).

Ticks. Male and female unfed nymph Rhipicephalus sanguineus (Latreille) ticks were purchased from the Oklahoma Tick Laboratory, Department of Entomology, Oklahoma State University, Stillwater, Oklahoma, USA. The ticks had been fed on laboratory rabbits and sheep for several generations and were considered free of vertebrate pathogens. Midgut and salivary gland from several unfed adult R. sanguineus were shown to lack microorganisms by examination with light and transmission electron microscopy. Ticks were maintained in small groups of 10 to 20 in 15 or 25 cm^2 clear plastic culture flasks with screw caps (Corning Glass Works, Corning, NY). The caps were modified to allow gas exchange by drilling a 5 mm diameter hole through the lid and covering the top with fine mesh Organza^{tm} silk (Classic Fabrics, Baton Rouge, LA). Tick-containing flasks were stacked in a glass

dessicator jar on a porcelain plate over a saturated solution of potassium phosphate monobasic (KH_2PO_4) (Sigma Chemical Co., St. Louis, MO.). This served as an acaridarium, which when placed into an incubator at 27°C maintains sufficient stable relative humidity in the range of 93.5-96%.[96] The light-dark photoperiod was controlled by an electric timer and incadescent light to provide 14 hours of light exposure per 24 hours. Ticks could be observed within the acaridarium without disturbing flasks or controlled climatic conditions.

Tick feeding procedure. Ticks were infested (fed) on dogs in two different instars (life stages). Unfed nymphs were fed on dogs experimentally infected with E. platys. Tick feeding capsules were attached to the lateral cervical region of dogs on 3 DPI with E. platys using general anesthesia. Anesthesia was induced by intravenous administration of thiamylal sodium (6-8 mg/lb) (Bio-tal^{tm}, Bio-Ceutic Div., Boehringer Ingelheim Animal Health Inc., St. Joseph, MO) and maintained by vented vaporization of halothane and oxygen administered by endotracheal intubation. Capsules consisted of double-thickness 4 inch diameter heavy weight orthopedic stockingette. Forty centimeter long tubular segments of stockingette were doubled over a 10 cm diameter ring placed between the two layers of stockingette. The ring was fashioned from

nonpyrogenic flexible sialastic-type tubing (C-Flex tubing, 1/8 inch. inside diameter; 1/4 inch outside diameter, Fisher Scientific, Baton Rouge, LA). The skin was shaved and lightly scrubbed with 70% ethanol which was allowed to dry thoroughly. Capsules were attached using 00 monofilament nylon surgical suture (Ethicon, Somerville, NJ) and surgical adhesive (cyanoacrylate ester adhesive, Loctite Co., Clevland, OH). There were no complications related to the procedure in any dogs and recovery from anesthesia was routine. Two E. platys-infected dogs and a control dog were each infested with unfed R. sanguineus nymph ticks. Both male and female ticks were placed within the attached tick capsules with access to exposed skin surface starting at approximately 100 hours post inoculation. Additional ticks were added to the capsules over the following two days (50 hours) until each dog was parasitized by approximately 100 nymph ticks. The unattached end of the stockingette could be opened to allow observation of ticks and collection of ticks following spontaneous detachment. Ticks were observed in feeding capsules two times each day. Dogs were observed and bled for platelet concentration and peripheral blood smear examination each day to verify E. platys infection and to correlate tick feeding with developement of parasitemia and thrombocytopenia. Tick

capsules were kept closed with elastic bands and masking tape, and overbandaged with porous gauze (Kling^{tm}, Johnson and Johnson Products, Inc., New Brunswick, NJ) and bandage material (Vetrap^{tm}, 3M company, St. Paul, MN). Replete (blood meal engorged) nymphs were returned to the acaridarium to allow ecdysis (molting) to the adult instar and maintaned for 1 month post molt. These unfed adult ticks exposed to E. platys-infected dogs as nymphs were then used in three studies. To study transmission of E. platys, 2 dogs were each infested by 32 male and female adult ticks placed in feeding capsules on 2 dogs. An additional dog infested with adult ticks that had fed on the uninfected control dog as nymphs, served as control in this portion of the experiment. Forty unfed adult ticks of both sexes that had fed on E. platys-infected dogs as nymphs were examined for morphologic evidence of E. platys organisms by light microscopy and transmission electron microscopy of ventriculocecal digestive system (midgut) and salivary gland. The midguts and salivary glands of an additional 30 male and female unfed adult ticks were examined by light microscopy for the presence of E. platys antigen using an immunocytochemical technique. To reactivate the invertebrate stage of the rickettsiae, all one month-old unfed adult ticks processed for microscopy were incubated at 37^{o}C for 2.5 days prior to

dissection.[55,56,58,61] Control ticks were treated similarly with each study of E. platys-exposed ticks.

Light, transmission electron, and immunocytochemical light microscopy techniques. Incubated unfed E. platys-exposed adult R. sanguineus as well as control ticks were dissected by securing the appendages and mouthparts in partially molten paraffin wax (Paraplast, Monoject, St. Louis, MO). With the aid of a dissecting stereomicroscope, the dorsal scutum was incisized circumferentially with an iris knife needle. Within 45 seconds following the initial stab incision, the carcass and viscera were covered in situ by several drops of cold (4^{o}C) 2% glutaraldehyde fixative in 0.25 M sodium cacodylate buffer (Ted Pella, Inc., Tustin, CA). Buffer pH was adjusted to 7.2 with (0.2 N) hydrochloric acid. Osmolality of 2% glutaraldehyde in 0.25 M sodium cacodylate was 694 milimoles per kilogram (mM/Kg) and of buffer alone was 466 mM/Kg as measured by vapor pressure osmometer (Wescor, Model 5500, Logan UT). The vetriculus, ceca (midgut), and portions of hindgut were dissected from either sex tick and placed in toto in a 4 ml stoppered glass shell vial (American Scientific Products, Harahan, LA) containing abundant cold fixative. The salivary glands were dissected and placed in separate vials of fixative. After fixing for 1 hour, the tissues were

washed in 0.25 M sodium cacodylate buffer 3 times for 5 minutes each. Tissues were post-fixed for 1 hour in 2% osmium tetroxide in 0.25 M sodium cacodylate buffer (pH 7.2), osmolarity 563 mM/Kg. Fixed tick tissues were washed in buffer and dehydrated in a graded series of ethanol once for 5 minutes each in 50%, 70%, and 80% ethanol. Two 10 minute treatments in 90% and then 95% ethanol followed. After three 10 minute treatments with absolute (100%) ethanol, the tissues were treated for 5 minutes in a 1:1 (v/v) mixture of 100% ethanol and propylene oxide, followed by two 5 minute treatments in 100% propylene oxide. The tissues were then placed in a 1:1 (v/v) mixture of propylene oxide and Dow combination 732 and 332 epoxy resin[97] (DER 7/3) (Electron Microscopy Sciences, Inc., Ft. Washington, PA). DER 7/3 was mixed in the following proportions: 10g Dow Epoxy Resin 332; 3g Dow Epoxy Resin 732; 7g dodecenyl succinic anhydride (DDSA), and 0.5g of 2,4,6, tri-dimethyl aminomethyl phenol (DMP-30). The resin mixture was satisfactory either when prepared 30 minutes prior to use as well as when used following several days storage at 0°C. Warming the room temperature resin for 15 minutes in a vented 60°C oven facilitated handling. All plastic and glass ware were predried in a 60°C oven. Tick tissues were infiltrated with resin in closed vials for 3 days. Infiltration vials

containing tissues were kept in a closed dessicator jar over a standard dessicant to control moisture from high ambient relative humidity. In some cases, tissue sample vials were opened and placed under vacuum to remove trapped air bubbles from the viscid resin. On the fourth day of tissue infiltration, vial stoppers were removed and the propylene oxide was allow to completely evaporate under a vented laboratory hood overnight.

For polymerization, resin-infiltrated midguts and salivary glands were removed from vials gently with 22 ga. hypodermic needle and placed on filter paper to remove excess resin. Tissues were oriented in Beem^{tm} capsules (Ted Pella Inc., Tustin, CA) containing fresh DER 7/3. First stage polymerization was carried out in uncapped capsules placed over dessicant and under low vacuum for 24 hours in 37^{o}C oven to cure the resin and to remove air bubbles trapped around the tissue. Resin in Beem capsules was then allowed to polymerize for 24 hours at 45^{o}C, followed by an additional 24 hours at 60^{o}C. Polymerized resin blocks were kept at room temperature (22^{o}C) for 24 hours prior to trimming. Sections for light and transmission electron microscopy were cut with glass or diamond knives on an ultramicrotome (Sorvall MT 500, Dupont) and floated onto distilled water. One micrometer thick "semithin" sections for light microscopy were picked

up on glass microscope slides and stained with new methylene blue/azure II stain for 2 minutes on a 60°C slide warmer. Slides were examined with a bright-field light microscope (Labrolux 12, Ernst Leitz, New York, NY) using a 100X oil immersion objective lens. Thin sections (60-90 nm thick) were picked up on uncoated 200 mesh copper grids, stained with uranyl acetate and lead citrate and examined under a Ziess EM 10 electron microscope.

Tick tissues used for immunocytochemical stain technique were dissected as described above, but the viscera were kept moist using 0.25 M sodium cacodylate buffer (pH 7.2) rather than fixative. Dissected tissues were placed in plastic tissue molds containing ornithine carbonyl transferase tissue medium used to prepare frozen sections (Tissue-Tek II, O.C.T. compound # 4583, Lab-Tek Products, Naperville, IL) and frozen by immersion in liquid nitrogen. The frozen salivary glands and midguts were stored at -70°C until sectioned for staining. Immediately prior to staining, tissue blocks were sectioned with a cryomicrotome (Kryostat, 1720, Ernst Leitz) and mounted on glass microscope slides. Air dried, thawed sections were then fixed in cold (4°C) acetone for 10 minutes and washed in phosphate buffered saline (pH 7.4) for 20 minutes. An immunocytochemical stain (avidin-biotin-glucose oxidase complex immunocytochemical

reagents, Vector Laboratories, Burlingame, CA) was used to demonstrate E. platys antigen in tick tissue using immune canine serum as the primary anti-E. platys antibody. Biotinylated secondary antibody (goat anti-cat immunoglobulin G with high (99%) cross reactivity to canine immunoglobulin G) (Vector Laboratories, Burlingame, CA) was applied following incubation of tick sections and primary antibody. In this immunoenzyme stain system, avidin-biotin-glucose oxidase complex was linked to the primary antibody by the biotinylated secondary antibody. A tetrazolium salt chromogen substrate was used to indicate presence of antigen. Frozen smears of E. platys-infected platelets prepared as described in Appendix A served as a positive control for antigen detection. The primary antibody canine immune serum was previously demonstrated to contain antibody to E. platys by immunofluorescent antibody test (Appendix A). Serum from a healthy dog with no demonstrable E. platys antibodies served as a negative control when substituted for the primary antibody. Tick tissues from ticks infested on noninfected control dogs served as negative tissue control. Following immunocytochemical staining, sections were counterstained for 20 seconds with methyl green/alcian blue stain. Sections were examined under a bright-field microscope with 100X oil immersion objective

lens.

RESULTS

Rhipicephalus sanguineus nymph engorgement coincided with the appearance of infected platelets and thrombocytopenia in dogs (Fig. 1). One E. platys-infected dog (F12) had inclusions within platelets on 6 DPI, the second dog (E32) begining on 7 DPI. Blood platelet concentration began to decrease on 8 DPI. In dog E32, a maximium of approximately 60% of circulating platelets contained E. platys inclusions or morulae on 9 DPI, while dog F12 had a maximal parasitemia of 40% on 10 DPI. By 12 DPI both infected dogs were markedly thrombocytopenic (less than 20.0 X 10^3 platelets/ul), and no inclusions of E. platys were observed on blood smears. The noninfected control dog (K32) did not develop E. platys infection as assessed by platelet count, blood smear exam, and serum antibody titer.

Both sexes of R. sanguineus nymphs infested on dogs readily attached to the cervical skin of dogs in tick feeding capsules and were feeding within 24 hours. As nymph ticks became blood-filled they increased in size and became darker brown. Nymph ticks fed for 8 days, with individual ticks becoming replete after feeding from 3 to 8 days following introduction into tick feeding capsules.

Replete nymphs were collected from tick feeding capsules after detachment, 3 to 8 days following infestation, and were placed in the acaridarium. All feeding ticks were recovered from tick feeding capsules by 8 days post infestation. Replete nymphs underwent ecdysis to the adult instar after 18 to 23 days in the acaridarium.

One month-old (post molt) male and female adult R. sanguineus ticks exposed to E. platys-infected dogs as nymphs attached to the naive uninfected dogs and control dog (16 each dog), and became engorged with blood. Within approximately 96 hours after being introduced into tick feeding capsules, ticks were partially engorged with blood and some adult ticks were observed exhibiting mating behavior. Feeding male ticks had detached and selectively reattached to dog skin in an inverted position between the dog and engorging female tick. The majority of ticks became replete after 8 to 10 days and then detached. However, some male ticks remained in capsules for longer than 18 days before being removed manually. As replete adult ticks detached they were returned to the acaridarium. Females became quiescent within 24 hours and oviposited in 3-5 days; larval ticks hatched from eggs laid by these female ticks but were not studied further.

The naive dogs infested with adult E. platys exposed ticks were monitored daily for 60 days, and failed to

develop E. platys infection as evaluated by platelet counts and blood smear examination. Serum collected from all dogs at 28 and 55 days after adult tick infestation (introduction into capsules) lacked anti-E. platys antibodies. However, the naive and control adult tick infested dogs developed E. platys infection when challenged 60 days post tick infestation by intravenous inoculation of E. platys. Anti-E. platys antibodies were detected in sera by IFA from all dogs collected 30 days post experimental E. platys challenge.

The dogs appeared to tolerate these experimental conditions well. Tick feeding capsules remained attached for the duration of the experiment allowing ticks opportunity to feed normally over a several day period. Dogs did not exhibit signs of discomfort from attachment of ticks or feeding capsules. Following periods of tick feeding, the capsules were removed from dogs by removing sutures and lifting the ring and capsule away from the skin. Mild to moderate moist dermal erythema that occurred at the site of capsule dettachment, became significantly clinically less apparent within 48 hours. Eventually the hair coat regrew and there was no clinically evident effects from capsule attachment.

No individual rickettsia or colony of E. platys was detected in incubated exposed unfed adult R. sanguineus

tick midgut lumen, epithelium, myoepithelium, or salivary gland as examined by light microscopy and transmission electron microscopy (Figs. 2-6). Sections of tick tissue processed and embedded in DER 7/3 resin provided the resolution necessary for evaluating the presence or absence of microorganisms by light and transmission electron microscopy. Evidence of E. platys antigens were not observed in frozen sections of midgut and salivary gland stained by the avidin-biotin-glucose oxidase complex immunocytochemical method. E. platys morulae in infected platelets were stained by specific chromogen label by immunocytochemistry.

DISCUSSION

Dogs failed to develop hematologic or serologic evidence of E. platys infection when fed upon by adult ticks exposed as nymphs to dogs experimentally infected by E. platys. Other molted adult ticks exposed to the same E. platys-infected dogs did not have evidence of rickettsia in midguts or salivary glands as examined by light microscopy, transmission electron microscopy, and immunocytochemistry. These findings indicate that R. sanguineus may not serve as biologic vector of E. platys. The methods used in this laboratory study approximated natural vector-host ecology, and were similar to techniques used in other successful tick transmission

studies of rickettsial disease. E. canis,[9] Anaplasma marginale, (A. marginale),[56,98,99] A. centrale,[51] and Cowdria ruminantium[54,100] have all been transmitted by various ixodid tick species fed as nymphs on infected animals.

Apart from the evolutionary adaptation of the family Rickettsiaceae for infecting and persisting within an arthropod host, many factors influence the suitability of ixodid ticks to function as vectors of rickettsial disease. These factors include species of tick, sex of tick, site of attachment to vertebrate host, duration of feeding, coordination of tick feeding with parasitemia, degree of host parasitemia, number of ticks feeding, life stage of feeding tick, parasite immune defense mechanisms of both vertebrate host and tick, and tick behavior related to mechanical transmission.

This study provided conditions suitable for R. sanguineus to feed on its natural host in a preferred topographical location[101] until the ticks were replete and detached spontaneously. Tick feeding capsules designed for this study were adaptated from previously reported methods used in different domestic animal species. This previously unreported method for use on dogs, proved to be satisfactory for feeding R. sanguineus on dogs. Clinically, the dogs showed no signs of

discomfort and did not disturb the capsules. All ticks fed undisturbed and collection of virtually all ticks introduced, indicated that feeding conditions appeared optimal. R. sanguineus fed on dogs in this study for their usual lengths of time from 3 to 8 days as nymphs and from 10 to 18 days as adults. Anaplasmosis has been transmitted between cattle by ixodid nymph ticks that fed on an infected calf for as little as 24 hours. These nymphs were transferred and allowed to feed to repletion on an uninfected calf. Following ecdysis these ticks transmitted anaplasmosis as adults when fed on a susceptible calf.[56] The duration of tick feeding in this study of E. platys infection exceeded that in other tick-borne disease transmission studies,[67,102] and would presumably provide ample opportunity for parasite transmission to occur based on similar studies.

Transmission of rickettsia can be influenced not only by length of tick feeding but also by timing of tick engorgement with presence and concentration of a pathogen in the vertebrate host. Thus coordination of tick feeding with development of initial E. platys parasitemia and thrombocytopenia was empirical, yet not without precedent in transmission studies of E. canis,[41,42] Cowdria ruminantium,[54,100] and A. marginale.[58] Ehrlichia canis infection of ticks was achieved by allowing R. sanguineous

to feed on febrile dogs when morulae were detectable in buffy coat smears.[41] Dermacentor sp. nymphs that fed on cattle with a high percentage of A. marginale infected erythrocytes (up to 50%) transmitted anaplasmosis more readily than ticks feeding during lower rates of parasitemia.[103] Adult ticks fed as nymphs on chronically infected anaplasmosis carrier cattle with no apparent parasitemia, however, were also infective for susceptible calves.[98] Dogs experimentally inoculated with E. platys in this study developed a marked parasitemia (60% and 40% respectively), which approached the highest levels previously reported,[7] and parasitemia coincided with engorgement of nymph ticks fed on E. platys-infected dogs. Whether a critical number of E. platys microorganisms is necessary for a dose infective for arthropod vectors is not known. By examination of blood smears, the initial E. platys parasitemia is the highest and presumably would result in the greatest amount of infectious material for feeding ticks. The intrathrombocytic form of E. platys, however, may not be the most infective for the appropriate arthropod vector. The amount of parasite in vertebrate host's blood and duration of feeding are important factors in infecting ticks with rickettsiae.[66] The cyclic nature of E. platys infection in dogs complicates the coordination of vector engorgement and parasitemia. A

threshold dose necessary to infect a certain percentage of ticks has been shown for Russian spring-summer encephalitis virus and Colorato tick fever virus.[5] This concentration of infective organisms can be affected not only by vertebrate host infection but also by conditions of tick digestion known as the "gut barrier". Microorganisms must escape the gut barrier to successfully infect the tick gut epithelium. The gut barrier includes luminal and intracellular digestive enzymes, variations in gut epithelial cell membrane permeability, possible limitations on cell-pathogen receptors, and invertebrate immune defense mechanisms.[5]

Ixodid ticks have extended feeding habits, which maximizes opportunity for contact with and ingestion of Rickettsiaceae.[48] The tick must overcome vertebrate hemostatic capabilities in order to feed over a several day period. When a tick feeds upon a vertebrate host, it attaches by cementing itself to the skin surface and creates a small pool of blood to imbibe by lacerating small superficial dermal vessels.[102] The vertebrate host initiates an inflammatory response mediated by vascular and cellular mechanisms.[102,104] Repair of small holes in vertebrate blood vessels begins with vascular contraction and platelet plugging.[105,106] Tick saliva has anti-hemostatic, anti-inflammatory and immunosuppressive

properties that permit prolonged feeding on host blood.[104] Other hematophagous arthropods such as mosquitoes and tsetse flies have anticoagulant factors in their saliva. Tick saliva contains apyrase, an enzyme that degrades ATP and ADP, promoters of platelet aggregation, to AMP. In addition, prostaglandin E_2 (PGE_2) in tick saliva[104] inhibits platelet aggregation stimulated by collagen and ADP.[104,107] Theis[102] suggested that the accumulation of leukocytes at the site of tick feeding may play an important role in transmission of organsims, especially ehrlichiae. In the case of E. platys, however, which is potentially transmitted by tick ingestion of infected platelets, sufficient accumulation of platelets at the site of tick feeding to allow uptake of organisms by platlelets could be inhibited by tick salivary apyrase and PGE_2. The intrathrombocytic form of E. platys may not be the most infectious for a hematophagus arthropod, and transmission may occur following feeding during some other stage of E. platys infection.

Although certain rickettsiae are vectored by multiple arthropods, there is usually a most suitable arthropod that serves for biological transmission.[38,40,68] In addition, infection of a particular tick instar may be required for transstadial transmission to occur. Influences that dictate whether a rickettsia is

transmitted transstadially and transovarially are not defined.[9,100] E. canis transmission by nymphs fed on infected dogs as larvae, adult ticks fed as nymphs, and adults feed as both larvae and nymphs have all been reported,[9] though not repeated in other transmission studies.[41,108] Three species of Amblyomma ticks, A. sparsum, A. astrion, A. cajennense transmit Cowdria ruminantium as nymphs fed as larvae on infected cattle and not by other instars. Amblyomma pomposum and A. lepidum meanwhile transmit C. ruminantium from larvae to nymphs and nymph to adults.[100] The differences may result from different techniques, but variable results in vector transmission studies occur often and may be influenced by rickettsial strain, tick species or strain, and tick instar. The role of larvae and of multiple interstadial tick infestation in transmission of E. platys by R. sanguineus were not examined.

Large numbers of feeding adult ticks can produce considerable edema and elicit pruritis which may contribute to disease transmission failure.[52] Introducton of too few ticks may result in transmission failure due to an insufficient dose of rickettsiae being introduced into the naive vertebrate host. Similar (and fewer) numbers of R. sanguineus have been used to transmit E. canis.[41,42] Naive uninfected dogs failed to develop E. platys

infection when fed on by adult R. sanguineus. Local dermal edema at tick feeding sites in this E. platys transmission study was mild and did not appear siginificant enough to elicit any sustained scratch response by dogs. Transmisson failure due to insufficient tick numbers alone is unlikely since E. platys organisms were not observed in ticks by light and transmission electron microscopy and immunocytochemistry.

Under natural conditions ticks may be disturbed during feeding only to continue feeding later, even on another host during the same instar, thus creating the potential for mechanical transmission of disease agents. Adult male ixodid ticks have an interrupted pattern of feeding. After partial feeding for about 2 days they detach and seek a female tick to mate with. They reattach while mating.[102] Once mating is completed, males may change hosts to seek another blood meal or mate again. Partial feeding and changing hosts increases the potential for mechanical disease transmission[66] (Hair JA, personal communication, 1987). There is some evidence to suggest nymph and adult male Boophilus annulatus (Say) transferred from one calf to another during feeding can mechanically transmit A. marginale.[50] Anaplasma marginale is transmitted not only by several species of tick but also mechanically by mosquitoes and horseflies.[48] The tick

feeding capsules used in this study prevented the ticks from changing hosts and potentially transmitting E. platys by mechanical means. The tick feeding capsules prevented the dog hosts from grooming, disturbing or crushing ticks and scarrifying skin, or from ingesting the arthropod. Rickettsia rickettsii can be transmitted to human beings by crushing infected ticks and subsequently contaminating scarrified skin or mucous membranes with rickettsiae.[109] Ingestion of R. sanguineus plays a role in transmission of Hepatozoan canis among dogs.[110] These mechanical methods of transmitting E. platys were not evaluated in this study.

Adult R. sanguineus fed on E. platys-infected dogs as nymphs were allowed to age one month post ecdysis to demonstrate biologic transmission and to ensure that mechanical or circumstantial transmission did not occur. Ticks can pick up rickettsiae but fail to develop a lasting infection.[5] Temporary survival of a microorganism ingested in a blood meal prior to its inactivation may simulate a true biologic vector-parasite relationship. The temporary survival of rabies virus in ticks has been reported but no true biologic maintenance or transmission of the virus is known to occur.[111]

Failure of E. platys transmission due to poor survivability of the ehrlichiae in R. sanguineus was

considered as a possibility. Longevity of rickettsiae in most arthropods is unknown and survival in an unfed tick does have some limits. Adult R. sanguineus have transmitted E. canis to susceptible dogs for up to 155 days following tick infection.[42] These ticks fed on acutely infected dogs as both larvae and nymphs. Animal transmission and morphologic studies similar to those used for this E. platys study were described for A. marginale and C. ruminantium using 1 month-old unfed adult D. andersoni and Amblyomma variegatum, respectively, that were exposed to infected calves as nymphs only.[54-56,61,103,112] A. marginale can survive in laboratory maintained ticks for three months or longer. In some cases 6 month-old ticks from this group transmitted anaplasmosis.[48] Rickettsia rickettsii has been demonstrated in 4 to 6 month-old adult D. andersoni ticks by fluorescent antibody stain.[66] These findings in other rickettsial transmission studies indicate 1 month post ecdysis would probably not be detrimental to biologic transmission.

Stimulated growth and development of rickettsial organisms as colonies occurs when unfed adult ixodid ticks are incubated for 2.5 days at 37°C.[61] In the present study, ticks examined by light microscopy, transmission electron microscopy, and immunocytochemistry were

incubated for 2.5 days at 37°C immediately prior to dissection, to stimulate rickettsial growth and increase colony density. Incubation was used to simulate arthropod feeding and to induce rickettsial reactivation, that is reported to occur by Rickettsia rickettsii,[5] Theileria annulata,[56] Babesia bovis,[56] E. canis,[41,52] E. phagocytophila,[41,52] as well as A. marginale[48,56] in ixodid ticks. Reactivation may be mediated by tick hormones.[5] At the completion of feeding and for several days following, the tick midgut is distended with concentrated blood. The mucosal epithelium is difficult to discern and the lumen often contains exfoliated epithelial cells in the process of blood meal digestion. Such conditions limit the volume of tissue that can be examined morphologically. Ultrastructure of tick midgut is less obscurred in the unfed tick.[56] Thus incubation was substituted for tick feeding in ticks used for morphologic studies to increase surface area of midgut examined and to provide favorable morphology of midgut epithelium. For adult R. sanguineus ticks fed on naive uninfected dogs in the E. platys transmission study, it was felt that attachment to dogs and feeding for several days under conditions approaching natural host-parasite ecology would reactivate a potential pathogen.

Detailed description of processing arthropod tissue

in DER 7/3 epoxy resin for light microscopy and transmission electron microscopy has not been reported previously. The method described was compared (Simpson RM, unpublished data, 1987) to infiltration, embedding and sectioning of ixodid tick midgut in Spurr's resin[113] and medium grade L. R. White resin.[114] DER 7/3 proved to be a subjectively superior resin compared to either Spurr's resin or L. R. White resin. DER 7/3 preserved tick tissue well and yielded consistently good sections for both light and electron microscopic examination. Dow Epoxy Resin 332 is a low viscosity resin[97] that infiltrated poorly permeable tick tissues. Addition of Dow Epoxy Resin 732 imparts softness and flexibility to conventional epoxides but does not result in loss of strength.[97] Sections withstood conditions of low pressure and bombardment by an electron beam on 200 mesh copper grids in an electron microscope. It was necessary to have extended infiltration times of 4 days with close adherence to dehydration, infiltration, embedding, and polymerization schedules to achieve satisfactory sections for evaluation. Any less degree resulted in incomplete infiltration or poor polymerization of the DER 7/3 resin.

Experimental dog susceptibility to E. platys infection was established 60 days post adult tick infestation by inoculation with E. platys. The lack of

disease transmission may indicate certain conditions of vector-parasite-host ecology were not met, or that E. platys is not transmitted by R. sanguineus. This study did not examine the possibility E. platys may be transmitted directly, or that other arthropod vectors serve to transmit E. platys.

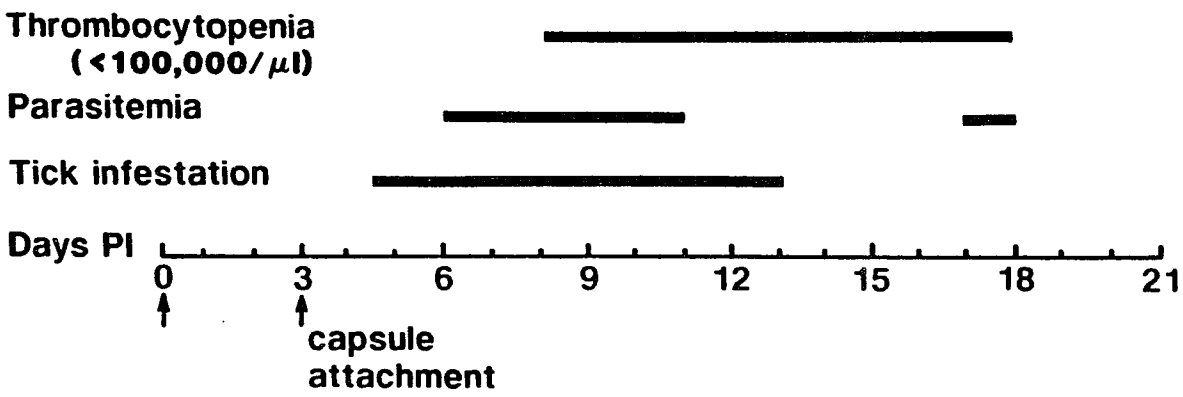

Figure 1. Temporal occurrence of nymph tick infestation with parasitemia and thrombocytopenia in E. platys infected dogs

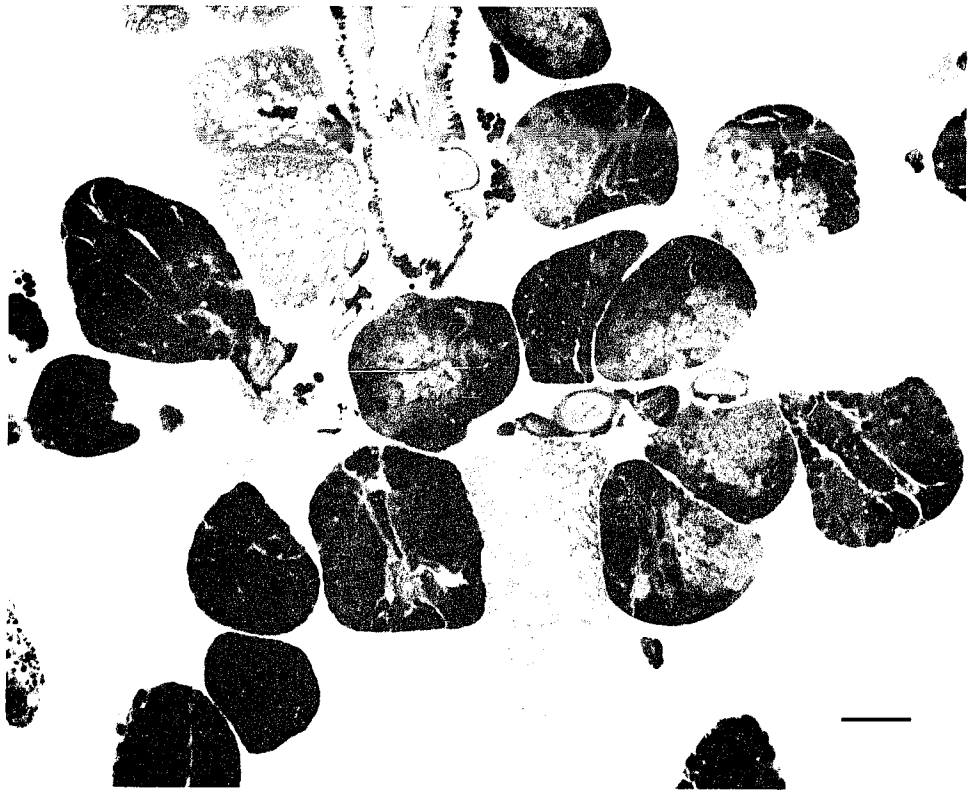

Fig. 2. Salivary gland acini, incubated unfed adult R. sanguineus tick exposed to E. platys infected dog. No organisms observed. Light micrograph, 1 um DER 7/3 resin embedded section, New methylene blue/azure II stain. Bar = 5.0 um.

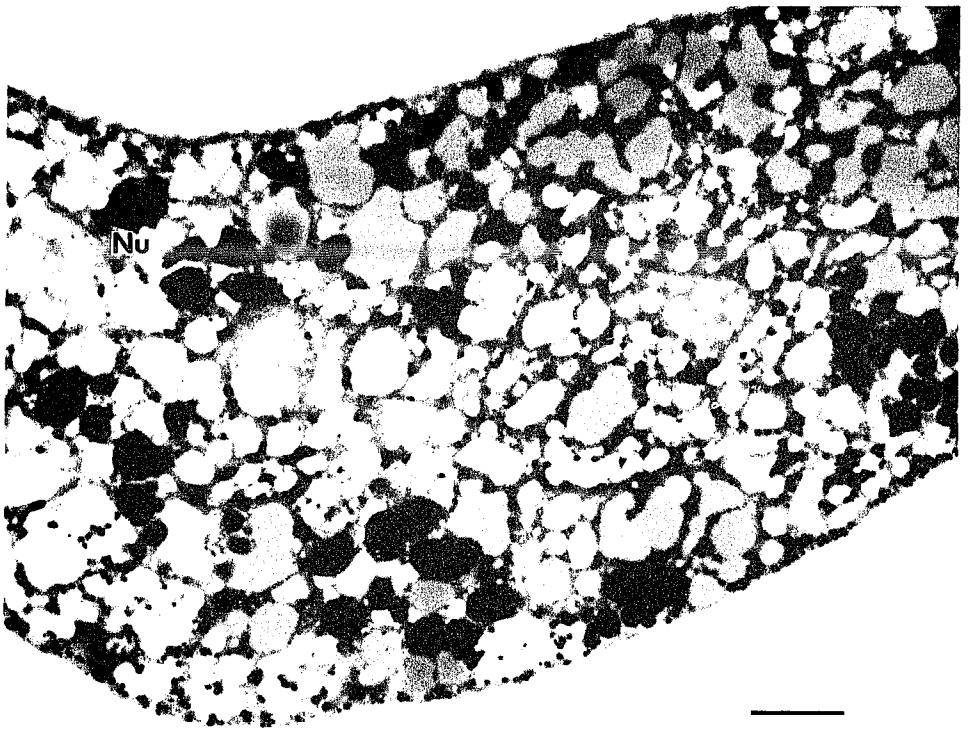

Fig. 3. Midgut, incubated unfed adult R. sanguineus tick exposed to E. platys infected dog. No organisms observed. Note individual epithelial cell membrane outline difficult to discern. Lumen not included in plane of section. Several types of cytoplasmic inclusions occur in epithelial cells depending on metabolic status. Note epithelial cell nucleus (Nu). Light micrograph, 1 um DER 7/3 resin embedded section, New methylene blue/azure II stain. Bar 5.0 um.

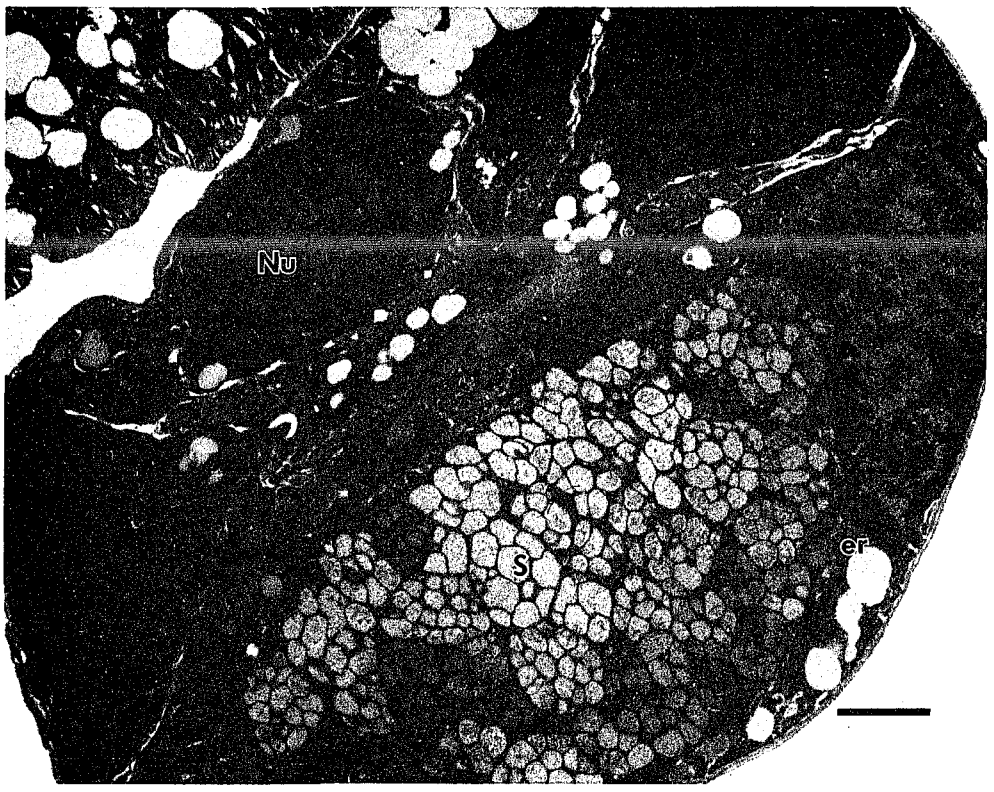

Fig. 4. Salivary gland acinus, incubated unfed adult R. sanguineus tick exposed to E. platys infected dog. No organisms observed. Note salivary gland cell nucleus (Nu), rough endoplasmic reticulum (er), and secretory structures (s). Transmission electron micrograph. Bar = 2.0 um.

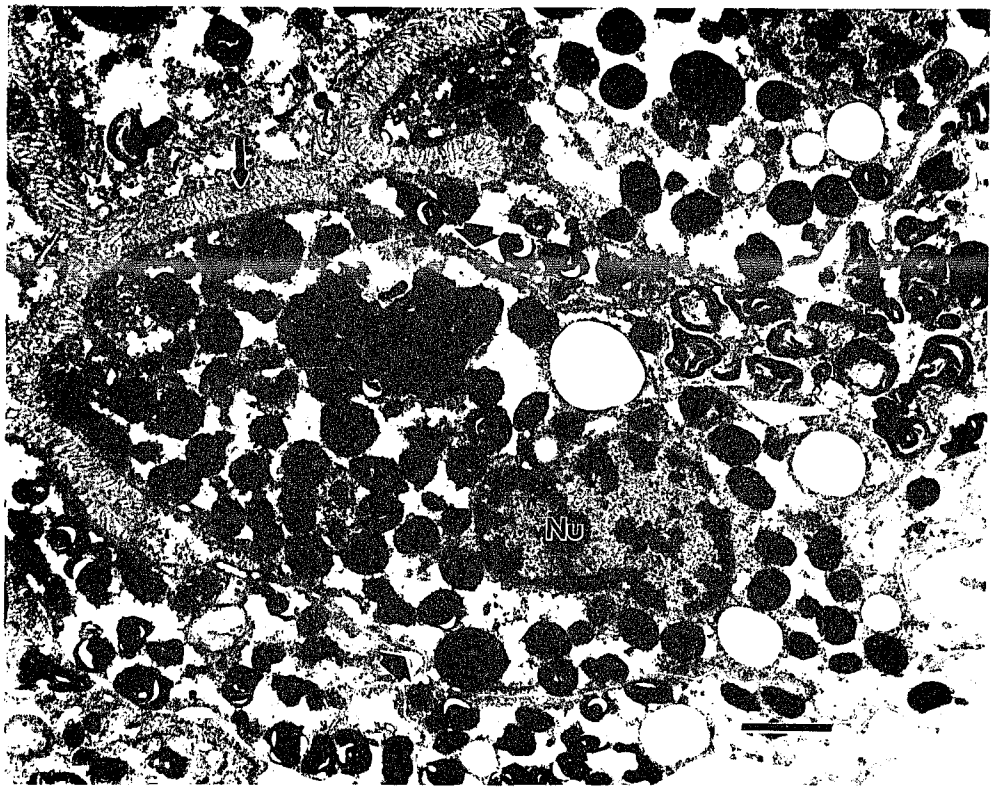

Fig. 5. Midgut epithelium, incubated unfed adult R. sanguineus tick exposed to E. platys infected dog. No organisms observed. Note luminal microvilli (small arrow), epithelial cell membrane (broad arrows), and nucleus (Nu). Cytoplasm contains numerous circumscribed electron dense granules. Transmission electron micrograph. Bar = 1.0 um.

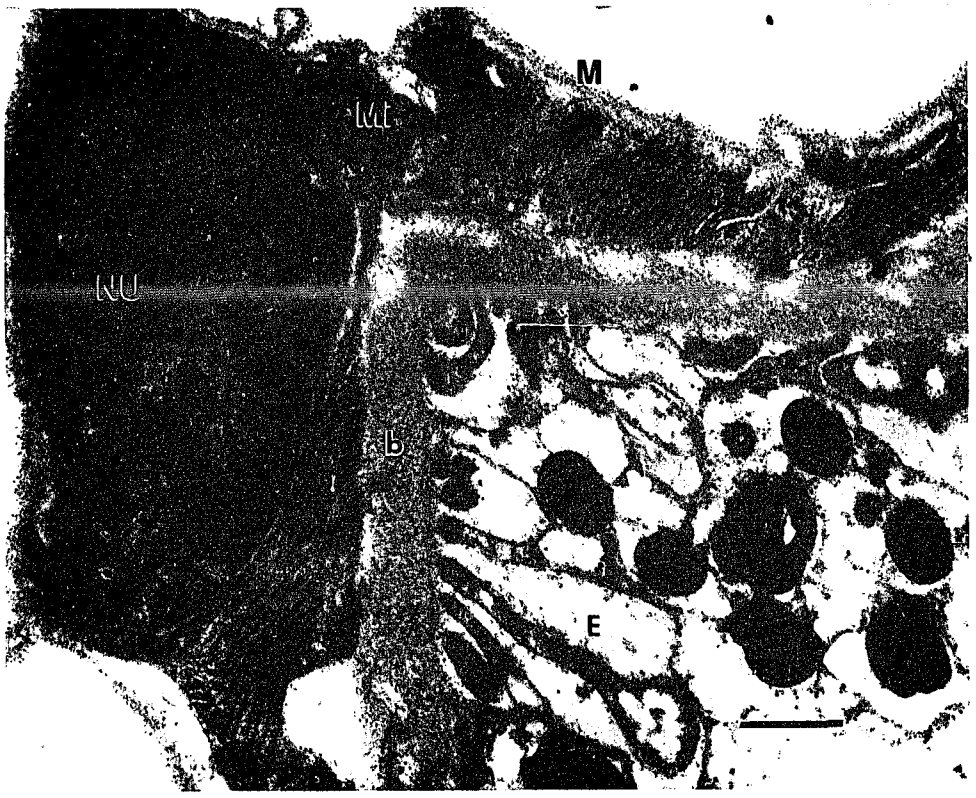

Fig. 6. Midgut myoepithelium (M), hemocoel surface, incubated unfed adult R. sanguineus tick exposed to E. platys infected dog. No organisms observed. Myoepithelial cell cytoplasm with regular light and dark cross striations, nucleus (Nu), and mithochondria (Mi). Note myoepithelium separated from midgut epithelium (E) by basal lamina (b). Transmission electron micrograph. Bar = 0.5 um.

CHAPTER IV

Immunocytochemical Detection of *Ehrlichia platys* Using an Avidin-biotin-peroxidase Complex Stain.

ABSTRACT

An avidin-biotin immunoperoxidase complex immunocytochemical stain procedure was optimized for detection of *Ehrlichia platys* antigens in canine blood platelets. The sources of specific anti-*Ehrlichia platys* antibodies for this assay were rabbit antisera, canine immune sera, and a murine monoclonal antibody. No *Ehrlichia platys* antigens were dectected when this stain was applied to formalin-fixed or acetone-fixed parffin-embedded tissues sections from dogs with *Ehrlichia platys* infection. An avidin-biotin-peroxidase complex procedure did detect *Ehrlichia risticii* antigens in acetone-fixed tissues from an infected horse, but did not in formalin-fixed tissue. Formalin treatment of *Ehrlichia platys*-infected canine platelets nullified positive immunoreactivity to ehrlichial antigens. Formalin-inactivated ehrlichial antigens in tissue sections from infected dogs were not unmasked by treatment with trypsin. Acetone fixation followed by paraffin embedding provided improved cytomorphology as compared to frozen tissue sections.

INTRODUCTION

Ehrlichia platys (*E. platys*) is a rickettsial organism that infects canine platelets.[7,12,17] Infection results in the appearance of *E. platys* inclusions or morulae in blood platelets (parasitemia) followed by cyclic episodes of thrombocytopenia that recur at 10-14 day intervals. Subsequent cycles of thrombocytopenia are preceded by progressively fewer numbers of infected blood platelets and many infected dogs appear to have spontaneous remission.[16] The blood platelet is the only tissue component unequivocably known to be infected by *E. platys*, but rare intracellular *E. platys* detected by transmission electron microscopy in tissue of infected dogs is evidence to support infection of other cell types (Chapter II). If and how *E. platys* infection persists in tissues of infected dogs, and how host immunity modulates infection is unknown.

The complete tissue and cell tropism of ehrlichiae is unknown. Specific histopathologic localization of ehrlichiae in tissue using conventional stains is less than satisfactory and *Ehrlichia canis* and *E. platys* can not be accurately observed in paraffin sections of tissues from infected dogs[37] (Chapter II). *Ehrlichia risticii* (*E. risticii*) has been demonstrated in horse intestinal lesions by modified Steiner silver stain,

immunofluorescent microscopy, and an immunoperoxidase immunocytochemical technique.[115]

Immunocytochemistry (ICC) has become a useful adjunct to problematic anatomic pathologic diagnosis by providing a system for a permanent tissue preparation with improved morphological preservation, allowing accurate localization of a wide variety of antigens in tissues by conventional light microscopy.[116,117] Immunocytochemistry has been used successfully on formalin-fixed paraffin-embedded tissue sections,[118] which is especially advantageous since such tissue processing methods are common in many laboratories and hospitals. There are many similarities between the principles of ICC and immunofluorescent microscopy. However, standardization of ICC methods has not advanced to the level of immunofluorescent microscopy, thus many pitfalls exist and rigorous controls of ICC specificity are required.[117]

The avidin-biotin-peroxidase complex (ABC) procedure is a multistep ICC process employing primary antibody specific for the desired antigen, biotinylated secondary antibody directed against the primary antibody, and preformed avidin: biotinylated horseradish peroxidase complex;[119] and it has been judged to be more sensitive when compared to the peroxidase anti-peroxidase technique.[118] Avidin is an egg white glycoprotein with high irreversible binding affinity for the small vitamin

biotin.[119] Avidin has four binding sites for biotin and acts as a bridge between biotinylated secondary antibody and biotinylated horseradish peroxidase enzyme. The enzyme contains multiple biotin moieties and can link additional avidin in a "lattice-like" binding arrangement, enhancing the sensitivity of the ABC method. Presence of the antigen is detected through enzymatic conversion of a suitable substrate to a visible reaction product.

The ABC method has been used previously to localize infectious microorganisms in tissues of infected animals. Rabies,[120] canine parainfluenza,[121] canine distemper,[121] canine adenovirus,[122] and Brucella abortus[123] are examples of antigens detected in paraffin embedded tissues of infected animals. In an effort to correlate lesion findings with presence of E. platys tissue antigens, an ABC immunoperoxidase stain technique was applied to tissues from dogs infected with Ehrlichia platys. To aid validation of this procedure in detecting ehrlichial antigens, the ABC method was used on Ehrlichia risticii-infected tissues as a positive control.

MATERIALS AND METHODS

Primary anti-ehrlichiae antibodies. Three different sources of anti-E. platys antibody were evaluated as immunocytochemical primary antibody. Ehrlichia platys antisera were produced in 4 rabbits. Canine immune sera

was collected 30 days post inoculation from 2 dogs (86R285 and U425) experimentally infected with E. platys. A murine anti-E. platys monoclonal antibody and a murine anti-E. risticii monoclonal antibody in murine ascites fluid were provided by Dr. R. E. Corstvet, Louisiana State University, Baton Rouge, LA. All primary anti-ehrlichiae antibody sources were determined to have specific immunoreactivity to respective Ehrlichia species by indirect immunofluorescent antibody assay and had antibody titers greater than 1:160.

Rabbit antisera to E. platys was produced in 4 Pasteurella-free, 4 month-old, either sex New Zealand White rabbits (Hazelton Labs, Vienna, VA). Serum was collected from all rabbits prior to inoculation, pooled, and frozen at -10^{o}C. The inoculum for rabbits consisted of aseptically prepared, washed, platelets containing inclusions or morulae from a dog (V-225) acutely infected with E. platys. Whole blood collected from this dog 12 days post E. platys inoculation (DPI) had 300.0 X 10^3 platelets/ul with approximately 30% containing E. platys organisms on Wright's stained blood smear examination. Aliquots of E. platys-infected platelets prepared as platelet-rich plasma (Appendix A) were washed and resuspended in sterile phosphate buffered (pH 7.4) 0.9% saline (PBS) at a concentration of 190.0 X 10^3

platelets/ul. The sterile, washed, resuspended E. platys-infected platelets were pulse sonified on an ice bath for 1 minute. Microscopic examination of Wright's stained smears of the sonicated platelet inoculum revealed the presence of E. platys microorganisms, numerous disrupted and few intact platelets. Rare leukocytes and erythrocytes had been observed prior to sonification.

Two protocols were used for production of E. platys immune sera in rabbits (Dr. Donald Dawe, Athens, GA, personal communication, 1986). Two rabbits were injected intravenously with an aqueous suspension of the above described inoculum via the marginal ear vein according to the following schedule. Inoculum dose was gradually increased (0.1 ml; 0.2 ml; 0.4 ml; 0.5 ml) over 4 consecutive days. Injections were repeated at a 3 week interval. These rabbits were given a third 0.5 ml intravenous booster injection of sonicated platelet inoculum. Serum was harvested 2 weeks later by vacutaner needle puncture of the central artery of the pinnae.

Another 2 rabbits received 2.5 ml of the sonicated E. platys-infected platelet suspension mixed equally (v/v) with complete Freund's adjuvant. Adjuvanted inoculum was prepared using a glass syringe and double hubbed needle to mix suspension until a drop would disperse evenly in water. The total 5 ml volume was administered subcutaneously as 0.5 ml doses in multiple sites along the

rabbits' dorsal trunk. Injections were repeated in 2 weeks using a suspension of sonicated E. platys-infected platelets mixed equally (v/v) with Freund's incomplete adjuvant. Subsequent booster injections without adjuvant were given to group B rabbits at 2 week intervals and serum was collected as described above. Sera were stored frozen at -10°C until used for ICC.

Prior to use in ICC, sources of anti-E. platys antisera were cross immunoabsorbed to either autologous or homologous canine platelets. Fresh or thawed aliquots of washed pelleted canine platelets from healthy uninfected dogs were incubated with test sera at 37°C for 30 minutes. After incubation, absorbed sera were centrifuged for 15 minutes at 10,000 X g (Microcentrifuge, Model 59A, Fisher Scientific, Baton Rouge, LA) to remove particulate matter and potential immune complexes. Sera were serially two-fold diluted 1:10 through 1:1280 in PBS to determine optimum concentration for immunoreactivity.

Cell and tissue preparations. Washed platelet-rich plasma smears of canine platelets containing inclusions and morulae of E. platys were prepared from acutely infected dogs. (Appendix A). Presence of E. platys organisms was confirmed in representative Wright's stained platelet smears examined by light microscopy. Platelet smears prepared from an uninfected healthy dog lacking

serum antibodies to ehrlichiae served as an E. platys-negative tissue control. Cultured murine P388D1 cells infected with E. risticii and uninfected P388D1 cells were prepared for staining by cytocentrifugation (Cytospin 2, Shandon Inc., Pittsburg, PA) of fresh culture derived cells onto glass microscope slides. Platelets and P388D1 cells were diluted in 1.75% bovine serum albumin. All cell smears were air dried, fixed in cold (4°C) acetone for 10 minutes, and stored at -70°C (Appendix A) until stained by ICC.

The following tissues were obtained from 1 E. playts-infected dog at 1 week intervals between 7 to 35 DPI: spleen, bone marrow, lymph node, liver, lung, kidney, and skin (Chapter II).[74] Tissues were stored as blocks of formalin-fixed, paraffin-embedded tissue until sectioned and processed for ICC. In addition, representative samples of organs were collected 12 DPI from an additional dog acutely infected with E. platys. Spleen, bone marrow, lymph node, liver, lung, kidney, and skin from this dog were processed by formalin fixation and paraffin embedding, by snap freezing in liquid nitrogen followed by acetone-fixation, and by acetone fixation followed by paraffin-embedding (Paraplast, Monoject, St. Louis, MO) in a tissue processor (LX300 Tissue Processor, Innovative Medical Systems Corp., Ivyland, PA). Formalin fixed tissues were immersed in 10% phosphate buffered (pH 7.2)

formalin solution for 24 hours at room temperature (22°C). Formalin-fixed paraffin-embedded tissues were processed to unstained tissue sections using standard procedure (Chapter II).[74] Frozen tissue samples were preserved by snap freezing in liquid nitrogen similar to technique described in Chapter III. Frozen tissues were stored at -70°C until sectioned (Kryostat 1720, Ernst Leitz, New York, NY). Frozen sections were fixed in 100% acetone for 10 minutes at 4°C prior to staining. A third group of representative tissue samples from the same E. platys-infected dog collected 12 DPI, were preserved by immersion in 100% acetone for 24 hours at 22°C.[124,125] Acetone-fixed specimens were dehydrated in 100% acetone for 3 changes of 15 minutes each, cleared in three changes of xylene (30; 45; 45 minutes) and embedded in paraffin (melting point 56-60°C) (Paraplast, Monoject, St. Louis, MO) in an automated tissue processor (LX300 Tissue Processor, Innovative Medical Systems Corp., Ivyland, PA). Paraffin-embedded tissues were sectioned (4-5 um thick) and picked up on glue coated (Elmer's glue, Borden Inc., Columbus, OH) glass microscope slides, allowed to air dry and stored (22°C) until application of ICC stain. Samples of spleen, lymph nodes, liver, lung, kidney, small intestines, small and large colons, and cecum from E. risticii-infected horses were obtained for ICC. All

tissues were processed for ICC by multiple methods, including freezing in liquid nitrogen, by immersion in 10% phosphate buffered (pH 7.2) formalin for 24 hours at 22°C, and by immersion in 100% acetone for 24 hours at 22°C. Formalin-fixed and acetone-fixed tissues were processed and embedded in paraffin as described above.

Immunocytochemistry Procedure. Immunocytochemistry methods (Dr. Pauline M. Rakich, Athens, GA, personal communication, 1987) were adapted for ABC localization of ehrlichiae tissue antigens and are outlined in Table 1. Avidin-biotin-peroxidase complex reagents were diluted as recommended by the manufacturer (Vector Laboratories, Burlingame, CA) in phosphate buffered (0.9%) saline (PBS--formula given in Appendix A). To enhance removal of paraffin from tissue, unstained paraffin-embedded tissue sections were dried overnight either in a 58°C oven or at 37°C. Paraffin was removed from tissues by washing slides containing tissue sections in 3 changes of xylene for 3 minutes each. Deparaffinized tissue sections were immediately rehydrated through ethanol using 3 separate changes of 2 minutes each in increasingly dilute alcohol, to tap water. Slides containing deparaffinized, rehydrated tissue sections were rinsed in PBS for 10 minutes at 22°C. Slides must be kept moist throughout the staining procedure. When stain reagents are applied to sections or cell smears, slides were kept in a humidity

chamber consisting of a petri dish lined with a moistened filter paper disc.

Endogenous tissue peroxidase activity was inhibited using an incubation step with hydrogen peroxide (H_2O_2) using various concentrations and time intervals. Tissue sections were subjected to H_2O_2 treatment ranging from 3% H_2O_2 for 5 minutes at 37° to 0.6% H_2O_2 in methanol for 30 minutes at 22°C (Table 1). Following H_2O_2 treatment, slides were rinsed in PBS for two changes of 10 minutes each at 22°C.

Formalin-fixed tissue sections were subjected to trypsin digestion following endogenous peroxidase block.[126] Tissues and petri dishes were warmed to 37°C prior to treatment with trypsin. Prewarmed formalin-fixed tissue sections were covered with 0.1% trypsin (Trypsin # T-0134 from porcine pancreas, Sigma Chemical Co., St. Louis, MO) and 0.1% calcium chloride (Sigma Chemical Co., St. Louis, MO) solution in distilled water, pH adjusted to 7.6 to 7.8. Trypsinization was carried out at 37°C for from 5 to 30 minutes. Trypsin activity was checked by immersing slides in cold (4°C) distilled water for 10 minutes followed by a 10 minute rinse in 22°C PBS. Tissues or cells preserved by freezing or acetone fixation were not subjected to trypsin digestion.

In the ABC method, nonimmune serum from the same

species used to produce the biotinylated secondary antibody is applied to sections for 20 minutes at 37°C to reduce nonspecific background staining. This nonimmune blocking serum was allowed to drain off sections and excess serum was blotted from around tissue or cell specimens with care to avoid contact with specimens or to allow them to dry to any degree. The various primary anti-ehrlichiae antibody sources were then individually applied to control and test tissues at dilutions ranging from 1:10 to 1:4000. Anti-E. platys antibodies were diluted in PBS. Anti-E. risticii monoclonal antibody was diluted in PBS containing 0.1% crystalline grade bovine serum albumin to further aid in control of nonspecific background staining. Slides were incubated with primary antibody either for 30 minutes at 37°C or overnight (approximately 20 hours) at 4°C. Slides were rinsed in PBS for 10 minutes at 22°C. To bind the immunoenzyme complex to the primary antibody, a biotinylated secondary antibody (antiglobulin) directed against immunoglobulin G of the species used to prepare the primary antibody was incubated with tissues. Since three different species of animals were used as sources of anti-ehrlichiae antibodies, each primary antibody required use of a different secondary antibody. Biotin-labeled anti-rabbit immunoglobulin made in a goat was used with primary antibodies (polyclonal serum) made in rabbits. Biotin-

labeled anti-cat immunoglobulin made in goat was used with anti-*E. platys* immune dog serum. Biotinylated goat anti-cat immunoglobulin (Vector Laboratories, Burlingame, CA) is produced for use with primary antibodies made in dogs and cross reacts (99%) with dog immunoglobulin G. Biotin-labeled anti-mouse immunoglobulin (H&L) made in horse was used with murine anti-*E. platys* and anti-*E. risticii* monoclonal antibodies. Slides were treated with the appropriate biotinylated secondary antibody for 30 minutes at 37°C followed by 10 minute rinse in PBS. Slides were incubated for 60 minutes at 37°C with avidin-biotin-horseradish peroxidase complex (ABC).

Avidin-biotin-peroxidase complex labeled antigen was identified by enzymatic conversion of a chromogen substrate. Avidin-biotin-peroxidase complex substrates tested included diaminobenzidine tetrahydrochloride (DAB) and 3-amino-9-ethylcarbazole (AEC). Because of numerous deposits of hemosiderin in many tissues from *E. platys* infected dogs and the color similarity of DAB to hemosiderin, AEC was more suitable for use on these tissues. Four miligrams of AEC (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 ml N,N dimethyl formamide (Sigma Chemical Co., St. Louis, MO). This mixture was stirred into 14 ml 0.1 M acetate buffer, pH 5.2 and 0.15 ml 3% H_2O_2. This chromogen was prepared and filtered

immediately prior to treatment of slides. Slides were incubated with AEC chromogen for up to 40 minutes at 22°C until a redish orange color developed.

Following incubation with chromogen, slides were rinsed for 5 minutes in tap water and counterstained with suitable stains, which included Mayer's or Gill's hematoxylin, or methyl green/alcian blue. Mounting medium (Coverbond synthetic resin, American Hospital Supply, McGraw Park, IL or Aqua-mount, Lerner Laboratories, New Haven, CT) appropriate for the particular chromogen depending on its solubility in solvents and water was used to coverslip tissue specimens. Slides were examined using a bright-field light microscope (Labrolux 12, Ernst Leitz, New York, NY).

Immunocytochemistry Controls. Assessment of antigen specific immunolabelling required fairly elaborate controls including tissues known to contain and lack the antigen sought. Platelet smears prepared from canine platelet rich plasma (PRP) from E. platys-infected and uninfected dogs served as controls for dog tissues. Slides containing E. risticii-infected and uninfected murine P388D1 cells were used as tissue control for staining experimentally infected horse tissues.

Additional control was provided by differences within test tissues and by variation in application of stain reagents. Portions of test tissues that lack antigen

serve to help distiguish specific staining from nonspecific background staining. Nonimmune pooled rabbit serum served as negative control when substituted for rabbit anti-*E. platys* serum on platelet smears and tissues from *E. platys*-infected dogs. Nonimmune canine serum from a healthy dog that lacked detectable antibody to either *E. platys* or *E. canis* served as a control when substituted for canine *E. platys* immune sera. A cocktail of several monoclonal antibodies directed against *Ehrlichia equi* antigens was used as negative control when substituted for the murine monoclonal antibody against *E. platys*. The murine anti-*E. platys* monoclonal antibody was used as a negative control when substituted for the anti-*E. risticii* monoclonal antibody on the horse tissues and P388D1 cells. Nonimmune control sera were cross immunoabsorbed as described by above methods. Occasionally, control was practiced by substitution of the primary antibody with PBS.

To determine the effects of formalin on ehrlichiae antigens, frozen acetone-fixed *E. platys*-infected canine platelet smears and *E. risticii*-infected murine P388D1 cells were post-fixed in 10% phosphate buffered (pH 7.2) formalin for 15 minutes at 22^{o}C followed by PBS rinse immediately prior to initiation of ABC stain.

RESULTS

Ehrlichial antigens were detected in cells and tissues by an avidin-biotin-peroxidase complex immunocytochemical stain. A summary of the staining results is presented in Table 2. Staining of controls was appropriately positive or negative in tissues during the same procedural application of ABC stain. Immunoreactivity of test tissue sections was absent when nonimmune serum was substituted for the primary anti-ehrlichiae antibodies. Ehrlichia platys antigens were detected in platelets preserved by freezing and fixing in acetone. As labeled by the ABC method, E. platys antigens appeared as single circumscribed usually eccentrically located densely stained area within platelets corresponding to chromogen color (Fig. 1). Canine E. platys immune serum (dog U425) diluted 1:10 provided most consistent antigen specific staining of E. platys in canine platelets. A high degree of nonspecific background staining hampered interpretation of cells or tissues stained with the rabbit antisera. The murine E. platys monoclonal antibody did not localize E. platys antigen and was deemed unsatisfactory for use with ICC.

E. risticii antigens were detected within horse intestine mucosal epithelial cell cytoplasm in frozen, acetone-fixed sections of cecum and in acetone-fixed, paraffin-embedded sections of large colon. Cecal and

large colon mucosal epithelial cell cytoplasm had small 2-4 um diameter densely stained areas corresponding to chromogen label. No staining of E. risticii was observed in other tissues. Mouse anti-E. risticii monoclonal antibody diluted 1:1000 gave the optimum staining for E. risticii tissue antigen in murine P388D1 cells and horse tissues.

Ehrlichial antigens were not detected by ABC technique in formalin or acetone-fixed tissues from the E. playts-infected dogs or formalin-fixed tissues from E. risticii-infected horses. Positive ehrlichial antigen immunoreactivity observed in canine platelets and murine P388D1 cells was markedly diminished or blocked when frozen, acetone-fixed platelet and P388D1 cell smears were post-fixed in formalin prior to application of ABC stain.

DISCUSSION

Immunocytochemistry methods applicable to detecting infectious agents in formalin-fixed paraffin-embedded tissues offer distinct advantages in identifying tissues infected with microorganisms because of wide application to routinely processed tissues. Utilization of acetone-fixed tissues for ICC provided tissue antigen localization of certain ehrlichial antigens, while retaining cellular morphology. The various sources of anti-ehrlichiae antibodies, which included polyclonal immune sera and

murine monoclonal antibodies used with an ABC stain in this study were unreactive with tissue sections routinely fixed in formalin from E. platys-infected dogs and E. risticii-infected horse.

Failure to detect ehrlichiae antigens by ABC staining of tissues other than platelets from dogs acutely infected with E. platys may result from either lack of antigen in the tissues or denaturization (masking) of the antigens during the fixation or embedding process.[118,121,127-130] Tissues may lack antigens because cells other than platelets are not infected by E. playts or because antigens diffused out of the tissue due to autolysis following tissue collection or during the embedding process.[117] Morphologic studies of E. platys infection have documented only rare organisms in cells other than platelets (Chapter II). Ehrlichia platys organisms were observed by transmission electron microscopy in a splenic cell of a dog infected for 14 DPI and in a pulmonary microvascular endothelial cell of another dog at 21 DPI. Immunofluorescent microscopy revealed the presence of E. platys antigens in mononuclear cells of spleen, liver, and bone marrow of dogs infected for 14 DPI that was interpreted to be evidence of either macrophage infection or phagocytosis of the organism or infected platelets (Chapter II). Ehrlichia platys may infect organs

sporadically, or predominantly during periods between the 7 day intervals examined in this study of E. platys infection, which might account for absence of organisms in ABC and silver stained tissue sections (Chapter II). While modified Steiner silver stain has been shown to stain tissue stages of E. risticii,[115] it has not been shown to stain E. platys. Machiavello stain, a histochemical stain used to stain rickettsiae, did not reveal tissue presence of E. platys as well (Chapter II). Even if E. platys does infect cells other than platelets, a minimal quantity of organisms may be needed in tissues to stain by ICC,[123] and this critical tissue mass of infectious ehrlichiae may not be achieved in infected dogs.

In contrast to E. platys, Ehrlichia risticii the causative agent of Potomac horse fever an acute febrile diarrheal disease,[131,132] has been demonstrated by transmission electron microscopic studies of intestinal cells of affected horses during acute infection.[133,134] In addition the organism has been observed in paraffin sections of intestine stained with modified Steiner silver stain.[115] Ehrlichia risticii antigen was detected in frozen acetone-fixed and acetone-fixed paraffin-embedded intestinal epithelium in an acutely infected horse. Positive ABC stain immunoreactivity for E. risticii was not observed in acetone-fixed tissues other than

intestines in this horse. The lack of ehrlichial antigens in tissues other than intestinal tract for an E. risticii-infected horse is similar to the lack of tissue antigens in E. platys-infected dogs. These findings would seem to indicate that polysystemic infection does not occur in these ehrlichial diseases.

Among the various sources of primary anti-E. platys antibody tested, the polyclonal canine anti-E. platys immune serum proved most suitable for use with the ABC method. Rabbit anti-E. platys polyclonal serum produced a greater degree of nonspecific background staining probably due to the high degree of accompanying antiplatelet antibodies. Initial purification of E. platys organisms from infected platelets using density gradient centrifugation and column chromatography[135] might have improved the antibody specificity of murine monoclonal antibody and rabbit antisera used for ICC in this study.

Antigens of E. platys or E. risticii were not detected by ABC method in tissues from infected dogs or horses that had been fixed in 10% phosphate buffered formalin solution. This suggests that formalin fixation denatures or inactivates ehrlichial antigens. Preservation of tissue antigen immunoreactivity is critically dependent on fixation.[128] Most T-or B-cell specific surface antigens,[124] canine paramyxovirus

antigens,[121] and others have been shown to be inactivated by fixation in formalin or other aldehyde fixatives.[127] Formaldehyde readily reacts with functional groups of biological macromolecules creating less reactive groups through intra- and intermolecular cross links.[136] Fixatives can also reduce tissue permeability for antisera,[128] and create stearic hindrance.[127] Formalin inactivation or masking of ehrlichial antigens was demonstrated in this experiment by post-fixing frozen acetone-fixed smears of E. platys-infected platelets and E. risticii-infected P388D1 cells in formalin. Positive immunoreactivity demonstrated in known infected cells by ABC stain was abolished in similar ehrlichiae-infected cells by exposure to formalin prior to application of ABC stain.

Treatment of paraffin embedded formalin fixed tissues with trypsin prior to application of immunofluorescent microscopy,[126,137] and ICC[122,138] (Gliatto JM. Ph.D. dissertation, Dept. of Veterinary Pathology, LSU, 1987) reagents has resulted in the unmasking of some formalin-inactivated tissue antigens. Trypsin is considered more efficient for revealing some antigens than pepsin, papain, or pronase.[139] Restitution of formaldehyde (formalin)-altered antigenicity is thought to consist of protease breakage of fixative-induced intermolecular cross links.[138] Trypsin treatment has also been reported to

reduce nonspecific background staining. Ehrlichia platys and E. risticii infected formalin-fixed tissues from acutely infected dogs and horses respectively, were subjected to a variety of treatments with trypsin. Trypsinization failed to return any formalin-fixed tissue antigens that may have been present to a structure recognizable by the primary antibodies tested. In some cases formalin-altered antigenicity has been unmasked by treatment of tissues with other proteases either alone or in combination with trypsin. Formalin-fixed tissues were not treated with any other protease solutions in this study. The possiblility that trypsin itself inactivates antigen[121] was not controlled directly by omitting trypsin treatment of tissue sections and additional study is needed to determine this effect. Immunocytochemical labeling of E. risticii in formalin-fixed intestinal epithelium from an infected horse was reported using an unspecified immunoperoxidase method.[115] This immunohistochemical method, however, was termed inadequate for immunostaining of E. risticii in a human histiocyte cell line that was fixed in formalin. Apparently E. risticii antigens were inconsistently immunolabeled in tissues treated with formalin, but these methods remain to be described.

This study examined different methods of tissue pro-

cessing and sources of primary anti-ehrlichiae antibodies, for detecting ehrlichial antigens in cells and tissues from E. platys-infected dogs and E. risticii-infected horses with an ABC immunoenzyme immunocytochemical technique and the aid of a conventional light microscope. The ABC method was used to detect E. platys antigens in frozen acetone-fixed infected canine blood platelets, and to detect E. risticii antigen in frozen acetone-fixed infected murine P388D1 cells. In addition E. risticii antigen was detected in frozen, acetone-fixed tissue sections of cecum and in acetone-fixed, paraffin-embedded sections of large colon from a horse infected with E. risticii. The method of acetone fixation preserved ehrlichial antigens and when followed by paraffin embedding provided improved morphologic preservation compared to frozen sections. Ehrlichiae antigen inactivation in routinely processed formalin-fixed paraffin-embedded tissues appears to be the result of formalin fixation rather than paraffin processing. Trypsin treatment of formalin-fixed tissues from ehrlichiae infected animals failed to result in detection of any antigens with avidin-biotin-peroxidase immunocytochemistry. Additional study is needed to determine if shorter treatment with formalin or combinations of fixation and enzymatic tissue treatment will provide a method of ehrlichia antigen localization in routinely processed tissues.

TABLE 1. Immunocytochemistry procedure using avidin-biotin-peroxidase complex reagents

Step 1)	Deparaffinize and hydrate tissue sections through xylene and graded alcohol series.[a]
Step 2)	Rinse in tap water 5 minutes.
Step 3)	Incubate specimen slides in 1.2-1.5% H_2O_2 in methanol for 30 minutes at 22 C.
Step 4)	Rinse in PBS, pH 7.4, for 20 minutes at 22 C.
Step 5)	Incubate prewarmed slides in 0.1% trypsin solution for 5-10 minutes at 37 C.[b]
Step 6)	Rinse in distilled water for 10 minutes at 4 C.
Step 7)	Rinse slides in PBS for 10 minutes at 22 C.
Step 8)	Incubate specimens with 1.5% normal nonimmune serum from the same species from which the secondary antibody is obtained, for 20 minutes at 37 C.
Step 9)	Blot excess serum from around cells or tissues.
Step 10)	Incubate specimens with primary antiserum diluted in PBS either 30 minutes at 37 C or overnight at 4 C.
Step 11)	Rinse specimen in PBS for 10 minutes at 22 C.
Step 12)	Incubate specimens with biotin-labeled secondary antibody (.45%) in PBS for 30 minutes at 37 C.
Step 13)	Rinse specimen slides in PBS for 10 minutes.
Step 14)	Incubate specimens with preformed ABC for 60 minutes at 37 C.
Step 15)	Rinse slides in PBS for 10 minutes at 22 C.
Step 16)	Incubate slides with chromogen (AEC, 40 minutes at 22 C).
Step 17)	Rinse in tap water for 5 minutes.
Step 18)	Counterstain and coverslip.

[a] Step omitted for frozen tissue.
[b] Step omitted for frozen tissue and acetone-fixed tissue.

TABLE 2.
Demonstration of *E. platys* antigens in cells and tissue after different processing methods

	frozen acetone-fixed	formalin-fixed paraffin-embedded		acetone-fixed paraffin-embed
Specimen	ABC	ABC	silver	ABC
PRP[a]	+	-[b]	ND	ND
spleen	-	-	-	-
bone marrow	-	-	-	-
lymph node	-	-	-	-
liver	-	-	-	-
kidney	-	-	-	-
lung	-	-	-	-
skin	-	-	-	-

+=positive
-=negative
a = platelet smears from platelet-rich plasma (PRP)
b = Cell preparations were post-fixed for 15 minutes in 10% phosphate buffered formalin but were not embedded in paraffin
ABC = avidin-biotin-peroxidase complex immunocytochemistry
silver = modified Steiner silver stain (results from Chapter II)
ND = not performed

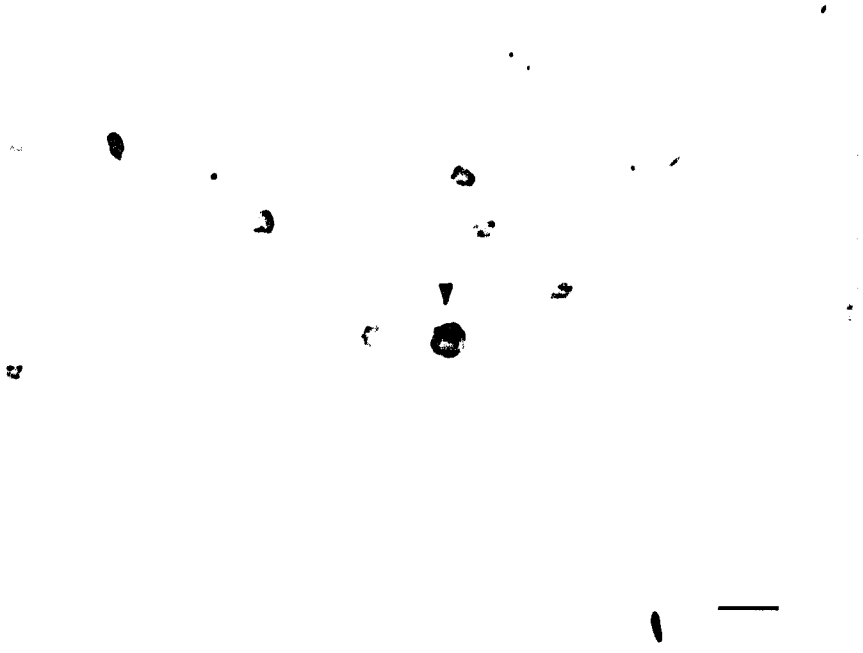

Fig. 1. Smears of washed platelets from E. platys infected dog. E. platys antigens immunolabeled in platelet (arrowhead) by immunoperoxidase method appear as eccentric circumscribed densely stained area. Other platelets in field are uninfected and lack immunolabel. ABC immunoperoxidase stain, methyl green/alcian blue counterstain. Bar = 2.0 um.

CHAPTER V

Summary And Conclusions

Mammalian infection by microorgansims of the tribe Ehrlichieae is being increasingly recognized, and previously unknown species have recently been described. Acute Ehrlichia platys infection in dogs causes predominantly subclinical disease, the hallmark of which is cyclic periods of thrombocytopenia associated with appearance of organisms within cytoplasmic vacuoles in blood platelets. Knowledge concerning this ehrlichial organism is important for comparison to other hemoparasites and to other causes of thrombocytopenia.

This series of investigations was designed to evaluate the pathogenesis of acute Ehrlichia platys infection and mechanism for transmission and maintenance of Ehrlichia platys in nature. Initially, dogs were experimentally infected with Ehrlichia platys from a naturally infected dog. There was multisystemic proliferation of lymphoid tissues and resident tissue macrophages in response to acute infection. Several organs contained hemorrhage or edema. Livers had hepatocellular vacuolation, small random multifocal hyperplastic nodules of Kupffer cells, and portal lymphangiectasia. All dogs surviving longer than 9 days

post inoculation developed parasitemia and thrombocytopenia. Following rapid dissappearance of platelets from circulation, blood platelet concentration increased over a 3 to 4 day period associated with increased megakaryocytopoiesis in bone marrow and spleen. Rare Ehrlichia platys infection of cells other than platelets, such as pulmonary microvascular endothelium, was observed by transmission electron microscopy. Lungs fixed by pulmonary airway perfusion of fixative lacked histopathologic lesions previously described in Ehrlichia platys infection. Ehrlichia canis and Ehrlichia platys produce similar morphologic lesions in acutely infected dogs.

Studies have demonstrated that both healthy and thrombocytopenic dogs often have serum antibodies to both Ehrlichia platys and Ehrlichia canis. This suggests that Ehrlichia canis and Ehrlichia platys, although different antigenically and in canine host cell tropism, share ecological niches that includes a common arthropod vector. Pathogen-free laboratory maintained Rhipicephalus sanguineus nymph ticks fed on dogs acutely infected with Ehrlichia platys failed to become infected and transmit Ehrlichia platys to susceptible dogs as feeding adults in this study.

The results of the vector study indicate that either

Ehrlichia platys is not transmitted by Rhipicephalus sanguineus or that conditions necessary for vector-parasite-host ecology were not met in the laboratory, effectively preventing transmission. Vector transmission trials utilizing other instars of Rhipicephalus sanguineus or other arthropods coordinated with different phases of canine infection must be investigated before concluding Ehrlichia platys is not vector-borne. Additional vector studies could utilize both the tick feeding methods used, and histotechnological methods for immunocytochemistry and for fixation and plastic resin embedding of ixodid tick organs for microscopy developed in this study.

An avidin-biotin-peroxidase complex immunocytochemical staining method provided a means for detecting ehrlichial antigens in cells and tissue sections with preservation of cytoarchitecture. Ehrlichia platys antigens were detected in frozen acetone-fixed infected canine blood platelet smears with polyclonal canine immune serum. Using a murine monoclonal antibody, Ehrlichia risticii antigen was detected in both frozen acetone-fixed sections and acetone-fixed paraffin-embedded sections of large intestines from a horse infected with the Potomac horse fever ehrlichia. The acetone fixation technique used, preserved ehrlichial antigenicity and provided good morphologic detail. Paraffin embedding acetone-fixed

tissue improved morphologic detail over frozen tissue sections and provided increased tissue surface area for examination. Ehrlichial antigens were not detected in formalin-fixed infected cells or tissues from infected dogs or horses examined by immunocytochemistry. Lack of ehrlichial antigen immunoreactivity in formalin-fixed tissues results from inactivation of antigen by formalin. Trypsin treatment of formalin-fixed tissues failed to unmask ehrlichial antigens for immunocytochemistry. Localization of ehrlichial antigens in routinely processed formalin-fixed tissues would be advantageous and additional study is needed to determine if an optimal method for unmasking erhlichiae antigens exists. Ehrlichia platys antigens were not detected in tissues from infected dogs using immunocytochemistry. Significant Ehrlichia platys infection of cells other than platelets detectable by immunocytochemistry may not occur.

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APPENDIX A

Cell Preparation For Immunofluorescence And Immunocytochemistry Microscopy

Ehrlichia platys-infected canine platelets for antigen slides were prepared by atraumatically collecting approximately 50 ml of blood by external jugular venipuncture from a 32 Kg splenectomized dog during initial plarasitemia of experimental acute E. platys infection. Blood was collected into a plastic syringe (Becton and Dickinson Co., Rutherford, NJ) containing one part 3.8% sodium citrate anticoagulant to 9 parts whole blood and mixed by gentle agitation. The anticoagulated blood was transferred to 50 ml plastic centrifuge tubes (Corning Glass Works, Corning, NY) and centrifuged (Beckman, model T-J, Palo Alto, CA) at 100 X g (700 rpm) for 15 minutes. The supernatant platelet rich plasma was harvested and centrifuged a 1000 X g for 10 minutes to pellet the platelets. The plasma (supernatant) was discarded. For E. platys antigen detection, platelets were washed 3 times by suspending the pellet in phosphate buffered 0.9% saline (pH 7.4) (PBS), and centrifuging at 1000 X g for 10 minutes. Washed platelets were diluted to a concentration of 100.0 X 10^3/ul in PBS containing 1.75% crystalline bovine serum albumin. Small drops of this

platelets suspension were gently smeared on glass slides precleaned with 95% ethanol and allowed to air dry. For Ehrlichia risticii antigen detection, cultured P388D1 murine macrophage cells were diluted in 1.75% bovine serum albumin and cytocentrifuged (Cytospin 2, Shandon, Inc., Pittsburg, PA) on to precleaned glass microscope slides. Platelet smears and P388D1 cells were fixed in cold (4°C) acetone for 10 minutes, air dried, and stored in an air-tight container at -70°C.

Fresh phosphate buffered saline was used throughout immunomicroscopy procedures. Each liter of phosphate buffered saline (PBS) contained:

1.15 g sodium phosphate monobasic (Na_2HPO_4)

0.20 g potassium phosphate monobasic (KH_2PO_4)

8.0 g sodium chloride (NaCl)

Salts were dissolved in ultrafiltered water (the equivalent of double glass distilled water) (Modulab Polisher, HPLC, Continental Water Systems Corp, New Orleans, LA). Sodium chloride (0.1 M NaOH) or sodium hydroxide (0.1 HCl) were used to adjust pH to 7.4.

Immunofluorescent Antibody Assay

Test sera were serially diluted and applied to thawed antigen and control slides. Slides were incubated a 37°C for 30 minutes, and then washed for 10 minutes in phosphate buffered saline (pH 7.2). Fluorescein-

congugated goat anti-dog immunoglobulin G (Cappel Laboratories, Cochranville, PA) diluted to 1:60 in PBS was applied to slides and incubated at 37°C for 30 minutes. Slides were then rinsed in PBS, and coverslipped and examined with an epifluorescence microscope (Ernst Leitz, Wetzlar GMBH 514662) for specific fluorescence.

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PRESENTATIONS AND ABSTRACTS

R. M. Simpson* and H. W. Taylor: Disseminated mycobacteriosis in a dog. 13th Annual Southeastern Veterinary Pathology Conference, May 17-18, 1985, Tifton, Georgia

R. M. Simpson,* E. C. Hodgin, and D-Y Cho: Disseminated extracephalic micronemiasis in a horse. 14th Annual Southeastern Veterinary Pathology Conference, May 17-18, 1986, Tifton, Georgia

S. D. Gaunt,* D. C. Baker, R. E. Corstvet, and R. M. Simpson: Platelet concentration and platelet aggregation in Ehrlichia platys or Ehrlichia canis infection. In Proceedings of 67th Conference of Research Workers in Animal Diseases, November 17-18, 1986, Chicago, Illinois, p. 54

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curriculum vita R. M. Simpson

PRESENTATIONS AND ABSTRACTS

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Veterinary Pathology

Title of Dissertation: Laboratory Investigation of the Pathogenesis and Vector Ecology of Canine Infection with Ehrlichia platys.

Approved:

Stephen D Gaunt
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Date of Examination:

July 1, 1988